University of Alberta

Enhancement of Low Temperature Germination and Early Seedling Growth in *Brassica napus*.

by

Cory Lee Nykiforuk

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Plant Molecular Biology and Biotechnology

Department of Plant Science.

Edmonton, Alberta

Fall 1996

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ISBN 0-612-18087-5

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Degree: Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Enhancement of Low Temperature Germination and Early Seedling Growth in *Brassica napus* submitted by Cory Lee Nykiforuk in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Molecular Biology and Biotechnology.

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Date: March 26, 1996.

This thesis is dedicated to my friends who supported me, to my family who never doubted me, and my wife who believed in me.

Abstract

Canola is seeded during the early months of spring when temperatures range from 5-10°C in Alberta. Generally, low temperature reduces germination and can result in poor early seedling growth. As emergence is a function of both developmental processes, these factors in turn can lead to asynchronous emergence and poor stand establishment. This can prolong the growing season, and thereby expose the crop to adverse environmental conditions during seed maturation and ultimately reduce yield. The objective of this study was to determine the effects of low temperature on germination and early seedling growth in *Brassica napus* cv. Westar. Two seedlots representing the extremes in germination potential were tested against numerous parameters including fresh weight, lipid mobilization, isocitrate lyase activity, and protein mobilization. The results indicated proper coordination between storage reserve mobilization, storage protein utilization, and development are paramount in determining the success of superior emergence at low temperature. One seedlot, Lendholm, was chosen for further study to examine the effect of low temperature on specific stages of germination and early seedling growth, to determine the cause of delays, to relate delays to seedling physiology, and then to recapitulate seedling emergence to give a clearer picture. Tests included examining CO_2 evolution, chlorophyll accumulation, isocitrate lyase expression, cruciferin mobilization, oleosin degradation, switches in developmental programs, and endogenous ABA levels. Thereafter, the effect of light during seed maturation was studied to relate environmental effects encountered during embryogenesis on storage protein deposition and ultimately on the ability of the seed to germinate. Poor seedling emergence at low temperature in *B. napus* cv. Westar is a function of temporal delays caused by thermal constraints during germination, and both decreased germination and reduced seedling growth, resulting from poor coordination of developmental processes. These effects can be modified by genotype or by the environment under which the

seed matures. In addition, the development of an artificial embryo system able to accumulate storage protein was explored.

Acknowledgements

Chapter 2. Acknowledgements: This research was supported by grants to A.M. J-F from Alberta Agriculture Research Institute and the Alberta Canola Producers Commission; additional funding was provided by the S.M. Blair Fellowship to C.L.N.

Chapter 3. A special thanks to Dr. M.L. Crouch (Indiana University, Bloomington), Dr. A. Huang (University of California, Riverside), and Dr. J.J. Harada (University of California, Davis) for the use of antibodies directed against cruciferin, oleosin, and ICL, respectively.

Chapter 4. The author is indebted to Dr. J.J. Harada (University of California, Davis) for the use of cDNAs recoginizing LEA 76, ICL, and COT 44. The author would also like to thank Dr. M. Raynal (University, France) for the use of B86 directed against a LEA type message.

Chapter 6. A special thanks to Dr. M.L. Crouch (University of,) for the use of antibodies and cDNAs against both cruciferin and napin.

I would also like to express my thanks and gratitude to my supervisor, Dr. A.M. Johnson-Flanagan for all of her guidance and patience. Along these lines, I would like to thank and acknowledge my supervisory committe consisting of Dr. D. Gifford, Dr. D. Cass, and Dr. A. Horak. Also my sincere gratitude towards my external defense examinor Dr. J.J. Harada.

With these acknowledgements I also wish to thank everyone in the Plant Biochemistry lab and Department of Agriculture, Foods, and Nutritional Science, and to those who have helped me in all aspects of graduate studies.

Table of Contents

Chapter 1. Literature Review..... 1

1.0. Introduction..... 2
1.1. Phylogeny..... 2
1.2. Historical Perspective..... 4
1.3. Current Status..... 4
2.0. Introduction To The *Brassica napus* Seed..... 5
2.1. Siliqua..... 5
2.2. Seed Structure..... 7
2.2.A. Seed Coat..... 7
2.2.B. Endosperm..... 9
2.2.C. Embryo.....10
2.3. Seed Reserves.....11
2.3.A. Carbohydrates.....11
2.3.B. Lipids.....11
2.3.C. Proteins.....12
2.3.D. Phytin.....13
2.3.E. Glucosinolates.....13
3.0. Embryogenesis.....14
3.1. Introduction.....14
3.2. Morphogenesis.....15
3.3. Embryo Maturation and Storage Reserve Deposition.....17
3.3.A. Lipid Biosynthesis.....17
3.3.B. Oleosins.....19
3.3.C. Storage Protein Biosynthesis.....21
3.4. Desiccation.....28
3.5. The Mature Embryo.....32
4.0. Germination.....34
4.1. Phases of Germination.....35
4.2. Low Temperature Effects During Germination...37
5.0. Early Seedling Growth.....40
5.1. Storage Reserve Mobilization.....40
5.1.A. Lipid Mobilization.....40
5.1.A.a. Lipases.....41
5.1.A.b. Glyoxysomes.....42
5.1.A.c. Glyoxylate Cycle Enzymes: Isocitrate Lyase and Malate Synthase.....44
5.1.B. Storage Protein Mobilization.....47
6.0. Artificial Embryogenesis.....49
6.1. Historical Perspective.....49
6.2. Microspore-derived Embryos.....50
6.3. Morphological, Biochemical, and Molecular Aspects.....50
7.0. Objectives.....52
8.0. Bibliography.....54

Chapter 2. Germination and Early Seedling Development Under Low Temperature in *Brassica napus* cv. Westar.....78

1.0. Introduction.....79
2.0. Materials and Methods.....81
2.1. Plant material.....81
2.2. Seed germination.....81
2.3. Isocitrate lyase assay.....81
2.4. Lipid determination.....81
2.5. Protein determination.....82
2.6. Protein analysis.....82
2.7. Statistical analysis.....82
3.0. Results.....84
3.1. Seed germination.....84
3.2. Seedling growth.....84
3.3. Extractable isocitrate lyase activity.....84
3.4. Lipid mobilization.....89

3.5. Total soluble protein.....92
3.6. Storage protein mobilization.....92
4.0. Discussion.....104
5.0. References.....110

Chapter 3. Storage Reserve Mobilization During Low Temperature Germination and Early Seedling Growth in *Brassica napus* cv. Westar.....114
1.0. Introduction.....115
2.0. Materials and methods.....118
2.1. Plant material.....118
2.2. Seed germination and seedling growth.....118
2.3. CO_2 determinations.....119
2.4. Chlorophyll determinations.....119
2.5. *In vivo* labelling.....120
2.6. SDS-PAGE.....120
2.7. Western blots.....121
2.8. Data analysis.....121
3.0. Results.....123
3.1. Seed germination.....123
3.2. Seedling growth.....123
3.3. CO_2 determination.....127
3.4. Chlorophyll accumulation.....127
3.5. *In vivo* labelling.....127
3.6. Western analysis.....127
4.0. Discussion.....141
5.0. References.....146

Chapter 4. Gene Expression During Germination and Early Seedling Growth Under Low Temperature Conditions in *Brassica napus* cv. Westar.....150
1.0. Introduction.....151
2.0. Materials and methods.....155
2.1. Plant material.....155
2.2. Seed germination.....155
2.3. RNA extraction.....155
2.4. Northern blotting; gel preparation and electrophoresis conditions.....156
2.5. Plasmid isolation.....157
2.6. Labelling of isolated cDNA fragments.....158
2.7. Hybridizing procedure and conditions.....159
2.8. SDS-PAGE.....159
2.9. Western blots.....160
2.10. ABA Analysis.....160
2.11. Germination in the presence of ABA or fluridone.....161
3.0. Results.....162
3.1. Seed germination.....162
3.2. Late embryogenesis abundant (LEA) transcripts.....162
3.3. Isocitrate lyase (ICL) transcripts.....168
3.4. COT 44 transcripts.....168
3.5. Western analysis.....168
3.6. ABA analysis.....168
3.7. ABA and fluridone effects during Low temperature germination.....172
4.0. Discussion.....176
5.0. References.....181

Chapter 5. The Enhancement of Low Temperature Germination in *Brassica napus* cv. Westar: Genotype Versus Phenotype.....185
1.0. Introduction.....186
2.0. Materials and methods.....188
2.1. Plant material.....188

2.2. Plant growth and selfing conditions.....188
2.3. Seed germination.....188
2.4. SDS-PAGE and Western blot analysis.....188
2.5. Bagged seed.....188
3.0. Results.....190
3.1. Germination potential of S_3 seed.....190
3.2. Seed weight.....190
3.3. Western blots.....190
3.4. Bagged seed.....196
4.0. Discussion.....203
5.0. References.....207

Chapter 6. The Accumulation of Storage Proteins in Microspore-Derived Embryos of *Brassica napus* cv. Topas.....209
1.0. Introduction.....210
2.0. Materials and methods.....212
2.1. Plant material and growth conditions.....212
2.2. Microspore production.....212
2.3. Protein analysis.....215
2.4. Western blot analysis.....215
2.5. RNA extraction and northern analysis.....216
2.6. Hybridization and stripping conditions.....216
2.7. Storage protein staining in fixed tissue.....217
2.8. MDEs *in vivo* labelling.....217
2.9. Cruciferin purification.....218
3.0. Results.....219
3.1. Total protein.....219
3.2. Western analysis.....219
3.3. Northern analysis.....230
3.4. Protein bodies in MDEs.....233
3.5. Cruciferin turnover.....233
4.0. Discussion.....242
5.0. References.....246

Chapter 7. Overview and Future Directions.....249
7.1. Introduction.....250
7.2. Normal emergence.....250
7.3. Germination.....251
7.4. Early seedling growth.....253
7.5. Low temperature emergence in *B. napus*.....254
7.6. Future directions.....256
7.7. References.....258

Appendix.....262

List of Tables

Chapter 2.

Table 2-1. Isocitrate lyase specific activity of Lendholm and Holmstrom seedlots at the optimal and suboptimal temperatures.

Table 2-2. Total protein content for Lendholm and Holmstrom seedlots at the optimal and suboptimal temperatures.

Chapter 3.

Table 3-1. Mean radicle lengths of seed germinated at 22, 10, and 6°C over the course of days after imbibition.

Table 3-2. Methionine incorporation rates during germination and early seedling growth at 22, 10, and 6°C.

Chapter 5.

Table 5-1. Fresh weight, % damaged seed, and % moisture content values obtained for parental L and H seedlots and the corresponding selfed seed populations, LS_0 and HS_0.

Table 5-2. Dry weight values of unbagged seed and bagged seed from 0, 12, and 28 DPA.

List of Figures

Chapter 1.

Figure 1-1. Phylogenetic tree of *B. napus* (Canola), and the cultivated varieties used in the present thesis (cv. Westar and Topas).

Chapter 2.

Figure 2-1. Comparisons in germination potential at 10, 6, and 2°C in the Lendholm and Holmstrom seedlots over the course of imbibition.

Figure 2-2. Fresh weight increases between the Lendholm and Holmstrom seedlots over the course of imbibition at 22, 10 , and 6°C.

Figure 2-3. Comparisons in extractable specific activity of ICL over the course of imbibition at 22, 10, and 6°C in the Lendholm and Holmstrom seedlots.

Figure 2-4. Comparisons in total lipid mobilization over the course of imbibition between the Lendholm and Holmstrom seedlots at 22, 10, and 6°C.

Figure 2-5. Protein profiles of the Lendholm and Holmstrom seedlots at 22°C. A. Over the course of imbibition (DAI). B. During specific developmental stages after germination (mm radicle length).

Figure 2-6. Protein profiles of the Lendholm and Holmstrom seedlots at 10°C. A. Over the course of imbibition (DAI). B. During specific developmental stages after germination (mm radicle length).

Figure 2-7. Protein profiles of the Lendholm and Holmstrom seedlots at 6°C. A. Over the course of imbibition (DAI). B. During specific developmental stages after germination (mm radicle length).

Figure 2-8. Protein profiles of the Lendholm and Holmstrom seedlots at 2°C over the course of imbibition (DAI).

Chapter 3.

Figure 3-1. Germination potential over the course of imbibition standardized in degree days at 22, 10, and 6°C in *B. napus* cv. Westar.

Figure 3-2. Fresh weight increases over the first 24 hours of imbibition at 22, 10, and 6°C in *B. napus* cv. Westar.

Figure 3-3. Fresh weight increases at 22, 10, and 6°C over the course of imbibition standardized as degree days in *B. napus* cv. Westar.

Figure 3-4. The evolution of CO_2 over the course of imbibition on the basis of equivalent degree days at 22, 10, and 6°C.

Figure 3-5. Chlorophyll accumulation on the basis of equivalent degree days at 22, 10, and 6°C in *B. napus* cv. Westar.

Figure 3-6. Cruciferin degradation at 22, 10, and 6°C at specific developmental stages past germination in *B. napus* cv. Westar.

Figure 3-7. Isocitrate lyase biosynthesis at 22, 10, and 6°C at specific developmental stages past germination in *B. napus* cv. Westar.

Figure 3-8. Oleosin degradation at 22, 10, and 6°C during seedling growth in *B. napus* cv. Westar.

Figure 3-9. Western blot analysis of oleosin degradation during early seedling growth ICL at 22, 10, and 6°C in *B. napus* cv. Westar.

Chapter 4.

Figure 4-1. Germination potential over the course of imbibition (DAI) at 22, 10, 6, and 2°C in *B. napus* cv. Westar.

Figure 4-2. Northern blot analysis of developmental molecular markers LEA76, B86, IL1, and COT 44 over the course of imbibition (DAI) in *B. napus* cv. Westar.

Figure 4-3. Northern blot analysis of developmental molecular markers LEA76, B86, IL1, and COT 44 during specific developmental stages past germination as measured by radicle length (mm) in *B. napus* cv. Westar.

Figure 4-4. ICL biosynthesis over the course of imbibition (DAI) at 22, 10, and 6°C in *B. napus* cv. Westar.
Figure 4-5. Endogenous ABA levels over the course of imbibition (DAI) in *B. napus* cv. Westar.
Figure 4-6. Germination potential in *B. napus* cv. Westar in the presence of 1 or 10 uM ABA, in the presence of 1 or 10 uM fluridone, or in ddH_2O at A. 22°C, B. 10°C, and C. 6°C.

Chapter 5.
Figure 5-1. Germination potential of parental L seed in comparison to LS_0 seed at 10, 6, and 2°C.
Figure 5-2. Germination potential of parental H seed in comparison to HS_0 seed at 10, 6, and 2°C.
Figure 5-3. Western blot analysis of parental L and H seed, and LS_0 and HS_0 seed for A. cruciferin and B. oleosin.
Figure 5-4. Germination potential of unbagged (control) and bagged (0, 12, and 28 DPA) seed at 22°C in *B. napus* cv. Westar.
Figure 5-5. Protein profiles on a per seed basis of unbagged (control) and bagged (0, 12, and, 28 DPA) seed in *B. napus* cv. Westar.
Figure 5-6. Western blot analysis of unbagged (control) and bagged (0, 12, and, 28 DPA) seed in *B. napus*. cv. Westar of A. oleosin and B. cruciferin subunits.

Chapter 6.
Figure 6-1. Schematic representation of the media conditions in which the MDEs were cultured in prior to the experiment and over the experimental time frame.
Figure 6-2. Protein profiles of isolated protein from MDEs in the CL and CD control conditions.
Figure 6-3. Protein profiles of isolated protein from MDEs in the dark pretreated conditions; DD(+) and DD(-).
Figure 6-4. Protein profiles of isolated protein from light pretreated MDEs maintained in the light; L(+) and L(-), or returned to the dark; D(+) and D(-).
Figure 6-5. Western blot analysis obtained from DD(+) and DD(-) MDEs against antibodies recognizing the α-subunits of cruciferin.
Figure 6-6. Western blot analysis obtained from L(+) and D(+) MDEs against antibodies recognizing the α-subunits of cruciferin.
Figure 6-7. Northern analysis of both cruciferin and napin transcript levels from total RNA extracted from all culture conditions and from zygotic embryos during maturation for comparisons.
Figure 6-8. Fixed MDEs stained for protein bodies. Included in the comparison are mature dry seed, L(+), DD(+), D(+), and control tissue (CL and CD).
Figure 6-9. Cruciferin turnover in purified extracts containing labelled cruciferin from MDEs (L(+) and DD(+)).

Appendix
Figure A-1. Cruciferin degradation over the course of imbibition (DAI) at 22, 10, and 6°C in *B. napus* cv. Westar.
Figure A-2. Oleosin degradation over the course of imbibition (DAI) at 22, 10, and 6°C in *B. napus* cv. Westar.

List of Abbreviations

ABA. Abscisic acid
ABRE. Abscisic Acid Response Element
ACC. Acetyl CoA Carboxylase
ACP. Acyl Carrier Protein
ATP. Adenosine Triphosphate
ATPase. Adenosine Triphosphatase
AT-rich. Adenine/Thymine-rich
B.C.E. Before the Common Era
BSA. Bovine Serum Albumin
Chl a. Chlorophyll a
Chl b. Chlorophyll b
CO_2. Carbon dioxide
C-terminus. Carboxy-terminus
cv. cultivar (cultivated variety)
D(+). Microspore-derived embryos cultured in the presence of osmotica and ABA after an initial light pretreatment.
D(-). Microspore-derived embryos cultured in the presence of osmotica after an initial light pretreatment.
DAF. Days After Flowering
DAG(s). Diacylglycerol(s)
DAI. Days After Imbibition
DAP. Days After Pollination
DD. Degree Days
DD(+). Microspore-derived embryos cultured in the presence of osmotica and ABA in the dark
DD(-). Microspore-derived embryos cultured in the presence of osmotica in the dark
ddH_2O. double distilled water
DEPC. Diethyl Pyrocarbonate
DNA. Deoxyribonucleic acid
DPA. Days Post Anthesis
EDTA. disodium ethylenediaminetetra-acetate
ELISA. Enzyme Linked Immunosorbent Assay
ER. Endoplasmic Reticulum
FACs. Fatty Acid Chains
FFA(s). Free Fatty Acid(s)
GAs. Gibberellins
H. Holmstrom seedlot
HAI. Hours After Imbibition
HPLC. High Pressure Liquid Chromatography
ICL. Isocitrate Lyase
IgG. Immunoglobulin G
kD(s). kiloDalton(s)
L. Lendholm seedlot
L(+). Microspore-derived embryos cultured in osmotica containing ABA in the presence of light after an initial light pretreatment.
L(-). Microspore-derived embryos cultured in osmotica in the presence of light after an initial light pretreatment.
LB. Luria-Bertani medium
LEA/*Lea*. Late Embryogenesis Abundant
MDE(s). Microspore-derived Embryo(s)
MOPs. 3-[N-morpholino]propanesulfonic acid buffer
MS. Malate Synthase
N-terminus. Amino-terminus
PBS. Phosphate Buffered Saline
ppm. parts per million
RER. Rough Endoplasmic Reticulum
RNA. Ribonucleic acid
SD. Standard Deviation
SDS. Sodium Dodecalsulfate
SDS-PAGE. Sodium Dodecalsulfate-Polyacrylamide Electrophoresis
SSC. (salt, sodium citrate) buffer

STE. (sodium chloride, Tris, EDTA) buffer
TAG(s). Triacylglycerol(s)
TBS. Tris Buffered Saline
TCA. Trichloracetic Acid
UV. UltraViolet
V. Volts
var. variety
2S. 2-sedimentation (Coefficient)
12S. 12-sedimentation (Coefficient)

Chapter 1. Literature Review.

1.0. Introduction.

Plants have been utilized for clothing, shelter, medicines, fuel, and have given rise to the unique atmosphere that has sustained life on earth (Stoskopf, 1981). Furthermore, it has been estimated that plant matter constitutes 70% of the human diet (Bewley and Black, 1994). For these reasons, plants are man's most important renewable resource. Bearing these factors in mind scientists' are expressing a growing concern over the ability to increase agricultural production in the face of population growth (Bongaarts, 1994; Schmidt, 1995).

Generally, the plant kingdom includes all living multicellular organisms possessing a cell wall and chlorophyll a (Barrett *et al.*, 1986). These organisms represent a very large, diverse, but specialized group. The classification of the plant used in the present study is *Brassica napus* L., commonly known as canola. Two cultivars of *B. napus* L., Westar and Topas, were selected for the present study. Canola varieties are based on tests that evaluate seed yield, days to maturity, height, lodging, disease resistance, percent oil content, percent protein content, glucosinolate content, and fatty acid composition (Canola Growers Manual, 1991). Westar, developed in 1982 by Agriculture Canada in Saskatoon, is the industry standard against which all other varieties are measured (Canola Growers Manual, 1991), and thereby served as an ideal variety to measure low temperature germination and early seedling growth in canola. Topas was developed by Svalof in Sweden in 1987 (Canola Growers Manual, 1991), and is ideal for tissue culture purposes allowing an unique opportunity to study embryogenesis.

1.1. Phylogeny.

The order *Capparales* (Cronquist, 1981) is characterized by plants possessing alternate leaves, flowers in racemes, calyx (a collective term for the sepals) and corolla (a collective term for the petals) with separate segments, superior ovary, and seeds that are high in oil reserves

Figure 1-1. The phylogeny of *B. napus* within the *Plantae* kingdom and the classification of the cultivars Westar and Topas. Derived from phylogenetic classifications according to Cronquist (1981).

Kingdom	***Plantae***
Division	***Magnoliophyta***
Class	***Magnoliopsida***
Subclass	***Dilleniidae***
Order	***Capparales***
Family	***Brassicaceae***
Genus	**Brassica**
species	***napus***
subspecies	***oleifera***
variety	***annua***
cultivar	**Westar**
	Topas

(Gill *et al.*, 1980). Of the five families in the order *Capparales*, the *Brassicaceae* family is the largest (3000 species) (Cronquist, 1981), and is characterized by flowers in terminal racemes with two pairs of sepals, four petals, six tetradynamous stamen, an exalbuminous seed, and a two carpellete ovary divided by an outgrowth of the placentas (Gill *et al.*, 1980; Cronquist, 1981). Canola possesses all of the aforementioned characteristics, and its phylogeny is shown in Figure 1.

1.2. Historical Perspective.

Brassicas date from the Oligocene era, 30-36 million years ago (Cronquist, 1988), and were cultivated as early as the 20th century B.C.E. in India (Canola Growers Manual, 1991). *B. napus* is an allotetraploid derived from an interspecific hybridization between *B. campestris* and *B. oleracea*, and therefore its existence must have originated in regions where these two species co-existed (Prakash and Hinata, 1980). From the geographic distribution of wild *B. campestris* and *B. oleracea* it is now believed that *B. napus* originated in the South-West European Mediterranean region (Prakash and Hinata, 1980).

1.3. Current Status.

Canola generates the most revenue, second to only wheat, amongst the major agronomic crops in Canada (Anonymous, 1994). On average, approximately 7 million acres are planted annually (Anonymous, 1994), and the large scale production in Canada ranks third in the international market behind China and India (Anonymous, 1990). In order for *B. napus* or *B. campestris* seed to be designated canola, it must first meet the requirements set out by the Canadian General Standards Board. Canola seed must have less than 2% erucic acid in the oil component and less than 30 µmoles/g of any mixture of glucosinolates in the solid component (Anonymous, 1990). Canola oil accounts for 63% of all vegetable oils processed annually in Canada (McDonald, 1990). The oils are not only used

extensively in edible products because of the low levels of saturated fats (6%) (McDonald, 1990), but are also used in cosmetics, industrial lubricants, plastics and for other commercial products (Anonymous, 1994). Likewise, the meal is also processed for use in livestock feeds because of the high protein content (37.7%) containing essential amino acids, in addition to minerals, vitamins, and fibre (Bell, 1993).

First, a general review of the structure and function of the various seed parts will give a macro-scale perspective of the whole seed environment. Next, an introduction to embryogenesis, followed by a section on germination and early seedling growth is required to attain the necessary background as it relates to the present study. Finally a brief description of artificial embryo systems will be reviewed.

2.0. Introduction to the *Brassica napus* seed.

A major adaptation to fertilization in the absence of an aqueous environment was the evolution of the seed. Not only did the seed protect the developing embryo during maturation, but it allowed the mature seed to subsist during adverse environmental conditions (Barrett *et al.*, 1986). Once favorable environmental conditions were met the seed (specifically the cotyledons in dicots) provided the necessary nutrients to the growing embryo until autotrophic growth could be maintained. A true seed has been defined as a fertilized mature ovule that contains an embryonic plant, with or sometimes without stored material, and is surrounded by a protective coat or coats (Kozlowski, 1972).

2.1. Siliqua.

In *B. napus* the seed matures within a silique (Latin for pod) formed from the remnants of the ovary. The silique consists of two carpels joined margin to margin, separated by a false partition, called the replum, which developed from the parietal placentae (Cronquist, 1981). The replum divides the one locule in two halves. Each silique varies in

the number of seeds it bears, but usually there are 14-40 seeds per silique (Downey *et al.*, 1975). The number of seeds per silique is correlated to the percentage of ovules with complete embryo sacs during fertilization (Bouttier and Morgan, 1993).

At maturity, the siliques undergo dehiscence, separating along the suture holding the carpels together, exposing the seeds attached to the replum (Esau, 1977). During seed maturation, large losses in seed yield result if excessive dehiscence occurs. This problem is known as shattering and provides one area for potential crop improvement. Generally, farmers swath crops prematurely in order to synchronize seed dehydration during maturation to reduce shattering (Cenkowski *et al.*, 1993; Coupe *et al.*, 1993), but this practice can result in green seed, which is economically undesirable (Ward *et al.*, 1992; Endo *et al.*, 1992). Biochemical and genetic mechanisms involved in dehiscence are currently under study (Coupe *et al.*, 1993; Coupe *et al.*, 1994).

During maturation the embryo requires nutrients such as nitrogen, phosphate, and sulphur for the biosynthesis of amino acids, nucleotides (for RNA and DNA synthesis), and oils. Two sources of mineral nutrients and assimilates for developing seeds are; (1) leaves or senescing vegetative organs and (2) fruit structures enclosing the seed (Hocking and Mason, 1993). In *B. napus*, Zhang *et al.* (1991) followed the translocation of labelled nitrogen proceeding from the old leaves to younger leaves, and eventually from younger leaves into the seeds. Nitrogen transportation within the silique occurred basipetally, and was translocated to the seeds in the same manner. Kullmann *et al.* (1991) found that the application of exogenous phosphorus and sulphur promoted nitrogen assimilation from the siliqua to the seeds inside. The silique not only serves as an important source of phosphorus and nitrogen, but has also been found to contribute zinc, magnesium and copper to developing seeds (Hocking and Mason, 1993). Apparently, the siliqua serves an important function, especially in situations where uptake of key nutrients ceases during seed filling, such

as drought. Evidence suggests the silique temporarily stores and subsequently redistributes nutrients to its developing seed (Hocking and Mason, 1993).

The siliqua are also capable of photosynthesis and are an important source of assimilates to the developing seeds (Chapman *et al.*, 1984; Rood *et al.*, 1984). The seeds also receive photosynthate from the leaves and stems, but the allocation of fixed carbon is variable in relation to reproductive development (Rood *et al.*, 1984 and references therein). During flowering, CO_2 assimilation is primarily derived from the leaves. Subsequently, the stem in conjunction with the leaves and siliqua, contribute photosynthate to the immature seeds. The siliqua become increasingly important as leaf senescence begins, and eventually during seed ripening, the silique is the principal site of CO_2 assimilation.

2.2. Seed Structure.

The mature seed of *B. napus* is relatively small, ranging in size from 2-3 mm. It usually has a brown seed coat. The seed is spherical with a reticulum along the median portion, and the surface is covered with microscopic pits (Musil, 1963; Berggren, 1981). The hilum, a scar marking the point at which the seed was attached to the mother plant via the funiculus (Bewley and Black, 1994), is concave, often 400-850 µm in diameter, and is covered around or above by smooth brownish grey cells similar to those of the testa (Berggren, 1981). A micropyle is also distinguishable near the hilum (Berggren, 1981). A cross section through a mature dry seed would reveal a multicellular seed coat, a relatively non existent endosperm (exalbuminous), and a large dicot embryo occupying the majority of the area.

2.2.A. Seed Coat.

The seed coat is the tough almost skin-like layer completely enclosing the embryonic tissue. The seed coat is often employed to

distinguish between genera and species (Bewley and Black, 1994), and the *Brassicas* are no exception (Downey *et al.*, 1975). The bitegmic seed coat is approximately 75 µm thick (Berggren, 1981), and is of maternal origin with the outermost cell layers (2-5) constituting the outer integument and the inner cell layers (up to 10) constituting the inner integument (Bhojwani and Bhatnagar, 1986). The outer integument consists of an epidermal layer and a palisade layer (Downey *et al.*, 1975). The outer epidermal layer is filled with mucilaginous material, consisting of pectin and cellulose, that will swell and tear when it comes into contact with water (Esau, 1977). The palisade layer is filled with heavy lignin deposits on the inner tangential and radial walls making this the strongest layer of cells in the seed coat (Esau, 1977; Bhojwani and Bhatnagar, 1986). The inner integument consists of a thin layer of crushed parenchyma that forms the pigment layer (Downey *et al.*, 1975), and therefore determines the color phenotype of the seed coat. The next cell layer is the aleurone layer, which is the outermost layer of the endosperm (Esau, 1977).

The seed coat is present early during embryogenesis, forming a partial boundary between the maternal and embryonic tissues. Some seeds possess a seed coat which can act as a barrier to the uptake of water (Omari, 1992; Todari and Negi, 1992; Serrato-Valenti *et al.*, 1993). In *B. napus*, the seed coat provides mechanical protection for the mature embryo. Unfortunately, the seed coat has not been extensively studied in canola, and therefore, other functions can only be speculated. However, in the legumes there are a number of functions aside from mechanical protection (Bhojwani and Bhatnagar, 1986; Bewley and Black, 1994) including, nutrient and assimilate transport to the developing embryo (Wang and Zheng, 1992; Offler and Patrick, 1993), nitrogen and carbon metabolite modification, regulation of water and gas exchange (Murray, 1987), and even pathogenic protection (Gijzen *et al.*, 1993). As the outermost layer of the seed it is the first tissue to come into contact with the environment.

Finkelstein (1994) reports that maternal effects have been documented in several species, including *Arabidopsis* (Korneef and Karssen, 1994), *Nicotiana tabacum* (Kasperbauer, 1968) and *Lycopersicon esculentum* (Taylor, 1979). Maternal influences, such as the delivery of plant growth regulators on the seed, could be derived from the mature seed coat or from material supplied to the developing embryo via the maternal vascular connection.

3.2.B. Endosperm.

The endosperm is derived from the fusion of a sperm nuclei and two polar nuclei (Lopes and Larkins, 1993) in *B. napus* (Groot and VanCaeseele, 1993). In *B. napus*, endosperm formation is initially free nuclear (Groot and VanCaeseele, 1993; Yadegari *et al.*, 1994), which means that nuclear divisions (karyokinesis) occur in the absense of cell wall formation (cytokinesis) (Esau, 1977; Lopes and Larkins, 1993). Compartmentation of the nuclei by cellularization occurs at approximately 16 DAP, and the endosperm has filled the embryo sac by 22 DAP except for a small central region (Groot and VanCaeseele, 1993). The endosperm cells contain lipids and starch, but also are a source of nitrogen, sulfur, phosphorus, and other minerals usually present as phytin (Greenwood, 1989) that are utilized by the developing embryo during maturation (Lopes and Larkins, 1993).

At seed maturity the outermost layer of the endosperm, the aleurone, is filled with a matrix of large and small lipid droplets surrounding proteinaceous bodies (Groot and VanCaeseele, 1993). *Brassica* is an exalbuminous seed, and therefore, the endosperm is absorbed by the developing embryo during maturation. Unlike most exalbuminous seeds, the aleurone layer persists until the seed matures, and this has raised questions about its function. Generally, in albuminous seeds the aleurone provides enzymes necessary to mobilize stored polysaccharides in the endosperm after germination (Ashford and Gubler, 1984). The endosperm

restricts germination in tomato (Leviatov *et al.*, 1994). Two other suggested functions may be control of oxygen uptake and/or control of water uptake or leakage from the embryo (Groot and VanCaeseele, 1993). Exogenous GAs have been shown to break dormancy by cell elongation forcing the radicle through the seed coat or endosperm (Salisbury and Ross, 1985). Therefore, the aleurone layer in canola and/or the seed coat may possess GAs that help break germination by initiating mobilization of stored carbohydrates. This is unlikely for *Brassicas* which lack an endosperm at maturity.

2.2.C. Embryo.

According to Martin's (1946) classification of seeds, *B. napus* embryos are classified as total (with respect to embryo/endosperm ratio), bent axile (with respect to embryo size, shape and position). Dicot embryos possess two cotyledons connected to a hypocotyl-root axis, with an apical meristem at both the shoot and root apex (Raven *et al.*, 1986). The entire embryo is bilaterally symmetrical, and its morphological organization can be divided into three domains (West and Harada, 1993). The apical domain includes the cotyledons, the shoot apex, and the upper portion of the axis. The central domain is comprised of the majority of the axis, and the basal domain includes the root apex. The shoot apical meristem terminates the epicotyl (also called the plumule), and the root apical meristem marks the end of the hypocotyl (also called the radicle) with the root cap terminating the root (Bhojwani and Bhatnagar, 1986; Raven *et al.*, 1986). These domains have been genetically delineated in *Arabidopsis* mutants that lack specific morphological structures related to each domain (Mayer *et al.*, 1991). Cross sections through the embryo axis reveal three different cellular zones organized spatially along a radial axis; (1) the outer epidermal layer of the embryo, (2) followed inwardly by the ground meristem or storage parenchyma (3) and the vascular procambium located centrally (Goldberg *et al.*, 1989, 1994).

2.3. Seed Reserves.

Embryos are filled with macro-molecules and nutrients that accumulate during development, and are stored in the desiccated embryo until the proper environmental conditions for germination are met. After germination, the storage products are utilized to support seedling growth until photosynthesis and autotrophic growth can be maintained. The major forms of storage products are carbohydrates, lipids, proteins, and to a small degree phytin (Bewley and Black, 1994). The amounts of these constituents in canola seed vary, but on average carbohydrates account for approximately 0.2-10%, lipids 42%, protein 37%, and phytin 3-6% (Bell, 1993). Canola seeds also include glucosinolates (14 umoles/g), tannins (1.6-3.1%), sinapine (0.6-1.8%), and a host of minerals (Bell, 1993).

2.3.A. Carbohydrates.

Carbohydrates are most commonly stored as two forms of starch, amylose and amylopectin (Bewley and Black, 1994). Amylose is a straight chain polymer, consisting of 300-400 glucose molecules linked by an α-1,4 glucosidic bond. Amylopectin consists of amylose chains linked via α-1,6 glucosidic bonds to produce branched molecules. Carbohydrates are not the major storage product in canola (Tykarska, 1987; Bell, 1993), compared to cereal grains or legumes, which contain 50-80% carbohydrates (Bewley and Black, 1994), and therefore, will not be discussed further.

2.3.B. Lipids.

Lipids represent an efficient form of stored energy, containing twice as much stored energy on a per-weight basis as starch or protein (Huang, 1992). The main storage form is TAGs, which are esters of glycerol and fatty acids (Bewley and Black, 1994). Greenhouse grown *Brassica napus* embryos contain approximately 95% TAGs, 1.2% DAGs, and 3.7% polar lipids (Perry and Hardwood, 1993). TAGs are stored in oil bodies

(Huang, 1992), which are spheres averaging 0.65 μm in diameter (Tzen *et. al.*, 1993). Isolated oil bodies are composed of 92-98% neutral lipids (TAGs), 1-4% phospholipids, and 1-4% proteins (Huang, 1992). The TAG fraction is a mixture of 6% saturated FACs (primarily palmitic acid), 58% monosaturated FACs (primarily oleic acid), and 36% polyunsaturated FACs (linolenic and α-linolenic acid) (Anonymous, 1994). The phospholipid and protein fraction form a half unit membrane (2-4 nm) around the stored oil with the proteins (oleosins) embedded in the oil body (Lee and Huang, 1991; Huang, 1992).

2.3.C. Proteins.

In *B. napus* two types of storage proteins, albumins and globulins, provide the necessary amino acids required after germination. According to Osborne's classification of storage proteins, albumins are water soluble, whereas globulins are soluble in salt solutions (Bewley and Black, 1994). In *B. napus* the 12S globulins are called cruciferin, and the 1.7S albumins are called napin (Lonnerdal and Janson, 1972; Finlayson, 1976; Crouch and Sussex, 1981; Rodin and Rask, 1990a).

Cruciferin accounts for approximately 50-60% of the total seed protein (Crouch and Sussex, 1981; Hoglund *et. al.*, 1992) with a molecular weight of 300 kD (Crouch and Sussex, 1981). It is characterized as a neutral glycoprotein (Dalgalarrondo *et al.*, 1986) composed of six subunits (Plietz *et. al.*, 1987), with each subunit composed of a heavy α-polypeptide (ranging from 29-37 kD) disulfide bound to a lighter β-polypeptide (ranging from 22-24 kD) (Dalgalarrondo *et. al.*, 1986; DeLisle and Crouch, 1989; Rodin and Rask, 1990a). Both proteins are sequestered and localized to protein bodies (Murphy *et. al.*, 1989b) deposited throughout the embryo (Hoglund *et. al.*, 1992).

Napin accounts for approximately 20-30% of the total seed protein (Crouch and Sussex, 1981; Murphy *et. al.*, 1989b; Blundy *et. al.*, 1991).

It is characterized as an alkaline 14 kD protein composed of two subunits, a heavy chain (9 kD) and a light chain (4 kD), bound together by a disulfide bridge (Crouch *et. al*, 1983; Ericson *et. al.*, 1986; DeLisle and Crouch, 1989).

2.3.D. Phytin.

Phytin is an important source of phosphate and minerals in the seed (Dalling and Bhalla, 1984; Bewley and Black, 1985). Phytin is an insoluble mix of potassium, magnesium, and calcium salts of phytic acid (*myo*-inositol hexakisphosphoric acid) (Greenwood, 1989). Phytin is localized within some of the protein bodies throughout the embryo, but phytic acid and its conjugates can display differential deposition in the embryo, for example calcium is highest in the protein bodies of the axis (Bewley and Black, 1994). Phytin represents anywhere from 50-90% of the total phosphorus reserve in the mature seed. It is catabolized by phytase (E.C. 3.1.3.26), releasing phosphorus and myo-inositol to the developing seedling during germination (Greenwood, 1989).

2.3.E. Glucosinolates.

To date there are approximately 100 naturally occurring glucosinolates identified in the *Brassicaceae* (Hoglund *et. al.*, 1991). Glucosinolates are thioglucosides linked to amino acid derivatives (Hoglund *et. al.*, 1991; Lenman *et. al.*, 1993 a and b), and are derived from methionine, phenylalanine, or tryptophan (Chavades *et al.*, 1994). In *B. napus* these include 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate (Anonymous, 1990). Glucosinolates are present in 2-5% of cells in the mature seed throughout the embryo (Hoglund *et al.*, 1991) especially in the outermost cellular layers (Drozdowska *et al.*, 1992), and are localized in myrosin grains within these cells (Lenman *et al.*, 1993 a and b).

Glucosinolates are hydrolized by myrosinase (thioglucoside

glycohydrolase E.C. 3.2.3.1) to evolve sulfate and numerous products including isothiocyanates, thiocyanates, nitriles, and others; some products are toxic. Although glucosinolate concentration in canola is relatively low because of traditional breeding, it is worth mentioning because of the role it may play in insect/plant interactions (Bodnaryk, 1992). It may function as a defence mechanism (Bones *et al.*, 1991; Bodnaryk, 1994), but the evidence for this is circumstantial (Bodnaryk, 1992).

3.0. Embryogenesis.

3.1. Introduction.

All vascular plants undergo an alteration from a haploid generation to a diploid generation to complete the life cycle (Barrett *et al.*, 1986). The haploid or gamete-producing generation is called the gametophyte, and the diploid or spore-producing generation is called the sporophyte (Raven *et al.*, 1986). In vascular plants (such as *Brassica*) the sporophytic generation predominates (Goldberg, 1988). The sporophytic generation leads to the development of gametophytes through sporic meiosis.

The microgametophyte produces the male gametes (sperm) housed in the pollen grain, and the megagametophyte produces the female gamete (egg cell) housed in the ovule (Bhojwani and Bhatnagar, 1986; Goldberg, 1988). In *Brassicas*, prior to the event of double fertilization (Goldberg, 1988; Lopes and Larkins, 1993), the male gametophyte (pollen tube) delivers the sperm to the egg cell through the style and stigma of the ovary via a process called "penetrative siphonogamy" (Bell, 1995), and will reach the ovary unless arrested by a mechanism of self-incompatibility (Guilluy *et al.*, 1991; Chasan, 1994) as displayed in *Brassica* species that outcross. One sperm cell fuses with the egg cell to result in the formation of a diploid zygote. The other sperm cell fuses with the endosperm nuclei (2n) to result in the formation of a triploid endosperm.

Once double fertilization is complete the resulting diploid zygote

will remain quiescent for a few days (Wilen, 1992) before undergoing further development. The period of zygote quiescence may involve polarization of the zygote within the embryo sac (West and Harada, 1993 and references therein) prior to the first division, and thereby predetermine the cellular fates of subsequent divisions. Indeed, in *B. napus* Tykarska (1976) describes polarization of the zygote with the nucleus embedded in dense cytoplasm at the apical end in comparison to the weakly vacuolated cytoplasm in the basal end.

Embryogenesis encompasses the period immediately after the fusion of gametes until the embryo has undergone desiccation and is developmentally arrested. During this period an array of morphological, cellular, and biochemical processes occur that establish the organization of the immature plant body and prepare the embryo for events leading to germination (West and Harada, 1993; Bewley and Black, 1994).

Embryogenesis can be delineated into three phases (Goldberg *et al.*, 1989; Kermode, 1990; West and Harada, 1993; West *et al.*, 1994). Early on, morphogenesis occurs, during which cellular divisions result in morphologically defined stages. The mid-stage of embryogenesis, maturation, is marked by cellular expansion and storage reserve deposition. During late embryogenesis the embryo undergoes desiccation and developmental arrest.

3.2. Morphogenesis.

During morphogenesis, (the first 20-24 DPA in *B. napus*) (Crouch and Sussex, 1981), the shoot and root apices are defined and cellular differentiation results in the formation of the embryonic tissue and organs (West and Harada, 1993). The zygote undergoes a sequence of defined cellular divisions (Tykarska, 1976, 1979). The initial transverse division results in the formation of a small chalazally oriented apical cell and a large elongated micropylarly situated basal cell (Tykarska, 1976). The apical cell will give rise to the embryo proper and the basal

cell will form part of the root apex and suspensor cell (Yeung and Meinke, 1993).

The development of the suspensor precedes that of the embryo proper (Yeung and Meinke, 1993). Prior to any further divisions, the basal cell elongates by redistributing the tonoplast (vacuolar membrane) to the cell plasma membrane (Tykarska, 1976). Next, the large basal cell undergoes a series of transverse divisions to result in the formation of the hypophosis and suspensor (Tykarska, 1976; West and Harada, 1993). The hypophosis is the precursor to the root cortex initials (Tykarska, 1979). The suspensor is a long filamentous structure 6-11 cells long (Tykarska, 1976), and forces the embryo proper into the nutrient rich endosperm (West and Harada, 1993; Yeung and Meinke, 1993). The suspensor functions as a site of hormone synthesis and transports nutrients to the developing embryo proper (Yeung and Meinke, 1993, and references therein). The suspensor reaches maximal cell number by the early globular stage, and senesces after the heart staged embryo in *Arabidopsis* (Mansfield and Briarty; 1991, 1992), a related crucifer.

The growth of the *B. napus* embryo from the apical cell is tri-modal, with rates of cell division increasing dramatically then diminishing (Tykarska, 1980). During stage 1, the apical cell divides to result in the formation of two tiers of octants (Tykarska, 1980). The apical cell undergoes two longitudinal divisions to produce a four-celled embryo (3-4 DPA), subsequently followed by a transverse division producing an octant (4 DPA) (Tykarska, 1976, 1980). Cells of the upper quadrant will form the cotyledonary region and embryo stem tip, while the hypocotyl will arise from cells in the lower quadrant (Tykarska, 1976). Next, cells within the octant undergo a periclinal division resulting in the formation of the protoderm (Tykarska, 1976) at the 16-celled embryo stage (5-6 DPA) (Tykarska, 1980). The protoderm is the precursor of the epidermis, and the histological detection of the protoderm marks the establishment of the globular staged embryo (West and Harada, 1993; Yadegari *et al.*, 1994) at

6-7 DPA (Tykarska, 1980).

The cells that comprise the young globular embryo contain the initials of all the histogens of the embryo axis (Tykarska, 1976). Further cellular divisions, including anticlinal cellular divisions of the protoderm and longitudinal and later transverse divisions of the internal cells, results in the transition from the globular stage (radially symmetrical) to the heart staged (bilaterally symmetrical) embryo. This marks the end of the second stage of embryo growth (Tykarska, 1980). This change consistently occurs around 18 DPA in canola (Fernandez *et al.*, 1991). Cell divisions in both the axis and developing cotyledon buttresses lead to the formation of a heart shaped embryo at approximately 20 DPA (Fernandez *et al.*, 1991). The cotyledons and axis can now be discerned, and histological differentiation reveals the procambium (which gives rise to the vascular tissue) and the ground meristem (which gives rise to the pith).

Stage 3 of embryo growth encompasses the period of growth from the heart staged embryo to the end of embryogenesis (Tykarska, 1980). After the formation of the heart staged embryo there is a period of uniform growth throughout the embryo. This is, however, followed by a period of cell divisions and rapid growth along the axis and cotyledons, in which the embryo elongates and results in the formation of the torpedo shaped embryo (Tykarska, 1979). During this stage, cells in the cotyledons and axis divide at different rates (Tykarska, 1979), with cells in the axis dividing rapidly leading to the formation of the torpedo stage embryo at 25 DPA, followed by cell expansion leading to the formation of the cotyledonary shaped embryo at approximately 30 DPA (Fernandez *et al.*, 1991). Concurrently, during maturation, storage reserve deposition takes place.

3.3. Embryo Maturation and Storage Reserve Deposition.

3.3.A. Lipid Biosynthesis.

Lipids, mainly in the form of TAGs, are derived from assimilates through an elaborate enzymatic pathway. Generally, reduced carbon is delivered to the seed via the phloem from other parts of the plant. In plants the biosynthesis of TAGs is carried out intracellularly within different organelles (Ohlrogge and Browse, 1995). The compartmentation of the substrates allows for some level of control over the rate of assembly of the TAGs. The onset of lipid deposition in *B. napus* occurs at approximately 21 DPA, increasing rapidly between 28 and 42 DPA, and levels off thereafter (Murphy *et al.*, 1989b; Kater *et al.*, 1991; Cummins *et al.*, 1993; Perry and Hardwood, 1993).

Within the proplastids, short 2 and 3 carbon precursors are converted to long chain fatty acids by the ACP-fatty acid synthetase complex (Slabas and Fawcett, 1992). ACP regulation in *Brassica* is tissue and temporally specific, appearing in the seed just prior to the onset of storage lipid biosynthesis (de Silva *et al.*, 1992; Slabas and Fawcett, 1992). ACP is nuclear encoded (Slabas and Fawcett, 1992) by a multigene family (some exhibit differential expression in the embryo) (de Silva *et al.*, 1990; Scherer *et al.*, 1992). Upstream sequencing of the ACP 5' end reveals palindromic sequences and an ABRE, which suggests trans-acting factors and responsiveness to ABA, respectively (de Silva *et al.*, 1992).

The rate limiting step in fatty acid synthesis is controlled by the enzyme Acetyl-CoA:ACP transacylase (Stumpf, 1987). This, therefore, provides one focal point for genetic engineering. By increasing or optimizing the activity of Acetly-CoA:ACP transacylase perhaps TAGs synthesis and deposition could be increased. Another point of regulation is controlled in part by ACC. The accumulation of its substrate, Acetyl-ACP, in the dark, is consistent with the light/dark control of fatty acid biosynthesis (Post-Beittimiller *et al.*, 1991; Ohlrogge and Browse, 1995). During embryogenesis in *Brassica*, ACC activity increases relative to the amount of lipid biosynthesis (Turnham and Northcote, 1983). However, once maximum lipid accumulation is reached, ACC is rapidly reduced (Turnham and

Northcote, 1983), and therefore, may centrally regulate the cessation of lipid synthesis (Slabas and Fawcett, 1992) by greatly reducing the substrates required for further lipid biosynthesis. Experiments designed to quantify the activity of ACC in the presence of ABA have not been carried out, but may provide some insight into the control of ACC expression during embryogenesis.

Other fatty acid synthesis enzymes have also been studied in *Brassica* including β-ketoacyl-ACP reductase (Sheldon, 1988), enoyl-ACP reductase (Slabas *et al.*, 1986; Kater *et al.*, 1991; Rafferty *et al.*, 1994), Acyl-ACP thioesterase (Loader *et al.*, 1993), and stearoyl-ACP desaturase (Hellyer *et al.*, 1992; Knutzon *et al.*, 1992; Slocombe *et al.*, 1992; Cummins *et al.*, 1993; Slocombe *et al.*, 1994). By defining the expression and kinetics of these enzymes, the overall role that each enzyme plays in fatty acid synthesis will be elucidated. This will provide researchers with an opportunity to manipulate fatty acid synthesis, and thereby alter the end products, resulting in the diversification of the oil seed crops (Ohlrogge, 1994 and references therein).

Fatty acids are delivered within the cell to the RER where they are covalently linked to a tri-glycerol backbone to produce DAGs required for phospholipid production, or converted to TAGs by diacylglycerol acyltransferase (E.C. 2.3.2.20) (Weselake *et al.*, 1991; 1993; Ohlrogge and Browse, 1995). As the TAGs are sequestered within the membrane of the RER, oleosin is believed to be co-translationally integrated initially into the bilayer of the RER, and subsequently transferred into the monolayer of the oil body, where the folding to the proper conformational structure takes place (Loer and Herman, 1993).

3.3.B. Oleosins.

Oleosins account for approximately 10% of the total seed protein (Huang, 1992; Cummins *et. al.*, 1993). Oleosins are a group of proteins

that confer special properties to the oil bodies (reviewed in Huang, 1992). These proteins are characterized by an amphipathic domain at the N-terminus, a long central hydrophobic domain, and a amphipathic alpha-helical domain situated at the C-terminus (Lee and Huang, 1991; Huang, 1992). The three structural domains result in a conformational state in which the C- and N-termini lie on the surface of the oil body and the highly conserved central region penetrates the oil body as an anti-parallel beta-strand, terminating in a proline knot (Huang, 1992). The observation of maize (a monocot) oleosin correctly being targeted to oil bodies in transformed *Brassica* (a dicot) (Lee *et al.*, 1991) suggests that the targeting element lies within the highly conserved central region (Tzen *et. al.*, 1990; Huang, 1992). The function of the oleosins, aside from providing structural stability during desiccation in the mature dry seed (Cummins *et. al.*, 1993), is postulated to act as a docking site for lipases during germination (Huang, 1992). To date, three isoforms exist in *B. napus*, and these seed isoforms are differentially expressed in a spatial manner within the embryo tissue in relation to the cotyledons and radicle (Cummins *et. al.*, 1993).

Oleosin accumulation has been shown to closely follow the accumulation of lipids in maize and *B. napus* (Tzen *et al.*, 1993), however, it has also been shown that storage oils accumulate prior to oleosin accumulation (Cummins *et al.*, 1993). These discrepancies reflect the need to improve the methodology in discerning differences in the expression between the oleosin isoforms in *B. napus* (Cummins *et al.*, 1993). Only then will further research delineate whether there is a concomitant integration of oleosin into the oil body during oil body formation (Hills *et al.*, 1993; Loer and Herman 1993) or the oleosins are imported into the oil bodies after formation, as suggested by Cummins *et al.* (1993).

Oleosins range in size from low to high molecular weight species (16-26 kD) (Tzen *et al.*, 1990) and are temporally specific, being expressed exclusively during seed maturation (Lee and Huang, 1991; Huang,

1992; Cummins *et al.*, 1993). However, an oleosin-like protein is also expressed in rapeseed pollen (Roberts *et al.*, 1993) reflecting its importance in the packaging of oils. Sequence comparisons based on nucleotides and amino acids between oleosins from varying species suggests that the isoforms are the result of a duplication event that took place prior to the divergence of monocots and dicots (Tzen *et al.*, 1990). *Brassica* oleosin contains putative regulatory elements in the 5' upstream region that are characteristic of seed-specific motifs and ABRE sites (Lee and Huang, 1991; Keddie *et al.*, 1994). The upregulation of oleosin expression by ABA is supported by observations of Wilen (1992), Keddie (1994), and Zou *et al.* (1995), in which oleosin transcripts increased in response to exogenously applied ABA and/or osmoticum. In carrot, two AT-rich upstream sequences of an oleosin gene were observed to interact with nuclear protein, and were also highly upregulated by ABA (Hatzopoulos *et al.*, 1990).

3.3.C. Storage Protein Biosynthesis.

During embryogenesis, storage proteins accumulate and are deposited in membrane bound organelles called protein bodies. These proteins will serve as a source of amino acids for the *de novo* synthesis of proteins during germination and early seedling growth (Callis, 1995). The two classes of protein prevalent in *B. napus* are the 2S albumin, napin, and the 12 S globulin, cruciferin (Crouch and Sussex, 1981; Rodin and Rask, 1990a).

Another class of proteins found in the cotyledons and axis of embryos, that accounts for up to 10% of the total seed protein in some seeds (Bewley and Black, 1994), are the seed proteinase inhibitors. These prevent the premature degradation of the stored nitrogen reserves. The function of the seed proteinase inhibitors is disputed (Dalling and Bhalla, 1984; Bewley and Black, 1994), however, these may serve as (1) a form of storage protein which is mobilized after germination (2) regulate

endogenous proteolytic enzymes during maturation, and (3) to protect or inhibit the seed tissue from insects or pathogens. One serine proteinase inhibitor has recently been isolated from *B. napus* seed (Ceciliani *et al.*, 1994), but its function remains to be defined. Furthermore, the overall percentage of seed protienase inhibitors in *B. napus* has not been determined to date.

Cruciferin is encoded by a gene family, and via transcription and translation, results in precursor peptides (Breen and Crouch, 1992). Based on immunological detection, the three precursors (P1, P2, and P3) range in size from 48 to 54 KD (Rodin and Rask, 1990 a and b). Each precursor is then processed to evolve its corresponding peptide chains. P1 gives rise to α1 (35-37 kD) and β1 (24 kD), P2 gives rise to α2/3 (31-29 kD), β2 (23 kD), and β3 (22 kD), and P3 gives rise to α4 (28 kD) and β4 (18 kD) (Rodin and Rask, 1990 a and b). Therefore, each precursor gives rise to a subunit pair (an acidic α-chain around 30 kD and a basic β-chain around 20 kD) (Rodin and Rask, 1990 a and b), which are bound to each other by disulphide linkages during processing (Dalgalarrondo *et al.*, 1986). Six subunit pairs bind via electrostatic and hydrophobic interactions (Rodin and Rask, 1990a) to complete the quantenary structure and form the mature hexameric protein (Dalgalarrondo *et al.*, 1986; Plietz *et al.*, 1987). The orientation of the subunits is proposed to be such that the strongly acidic C-terminal of the α-chain is situated at the surface of the molecule, whereas the hydrophobic C-terminal of the β-chain would face inward near the centre of the molecule (Plietz *et al.*, 1987; Robin *et al.*, 1991). Therefore, the hydrophilic exterior would protect the domains from the external aqueous environment, and the hydrophobic interior would stabilize the molecule by hydrophobic interactions between the six subunits.

The cruciferin cDNA clones pCRU1, pC1, and pC4 are thought to encode P1, P2, and P3, respectively (Simon *et al.*, 1985; Sjodahl *et al.*, 1991; Rodin *et al.*, 1992). Characterization of the P2 subfamily via Southern

blot analysis revealed the subfamily is encoded by a small gene family of five members (Breen and Crouch 1992). All members of the family exhibit high sequence homology extending into the 5' and 3' untranslated regions, and display similar restriction sites and intron positions. This suggests that the five member family is the result of gene duplication and not functional specificity (Breen and Crouch, 1992). Upstream sequence analysis revealed that all members contain a concensus promoter element (TATAAATA) 57 bp upstream of the initiation codon, and a concensus translation start site (AACAAUGGC).

In contrast to other species such as *Vicia faba*, *Glycine max*, and *Gossypium hirsutum*, which have only two types of globulin genes (Chlan *et al.*, 1986; Wobus *et al.*, 1986; Nielsen *et al.*, 1989), the *Brassicaceae* have three different cruciferin genes or gene subfamilies (Sjodahl *et al.*, 1991). Based on the alignment of deduced amino acid sequences in comparison to these other species the 12S globulin genes of the *Brassicaceae*'s evolved in two steps (Sjodahl *et al.*, 1991). Initially the ancestral 12S genes were duplicated to give rise to the present-day pCRU1 genes, and to a common ancestor of the pC1 and pC4 genes (Sjodahl *et al.*, 1991). The latter ancestor underwent another duplication event to result in the present subfamilies encoding the P2 and P3 precursor proteins.

Genes encoding napin give rise to a 20 kD precursor polypeptide containing a signal sequence that is removed during transfer of the protein into the endoplasmic reticulum (Ericson *et al.*, 1986). Two highly negatively charged regions, 7-8 residues long, that are common to the napin genes are located just before the small subunit at the amino-terminal end and within a stretch of residues between the small and large subunit (Ericson *et al.*, 1986; Josefsson *et al.*, 1987). These two repeats carry nearly all of the negative charges that are contained in the processed regions of the precursor, and thus are believed to play a role in processes associated with translocation, intracellular transport and\or deposition into protein bodies. These regions may also serve as signals

in the proteolytic steps necessary in the generation of the mature napin. Once processing is complete the heavy and light polypeptide chains are bound to each other by two disulfide bridges (Ericson *et al.*, 1986).

Napin is encoded by a gene family consisting of at least 16 members (Scofield and Crouch, 1987; Blundy *et al.*, 1991). The napin gene family can be split into two expression classes; products arising from the gNa class peak at 27-33 DPA and the other major class peaks at 37 DPA (Blundy *et al.*, 1991). Published napin sequences show a high degree of homology in both the coding and flanking regions (Ericson *et al.*, 1986; Scofield and Crouch, 1987). Napin genes cloned to date lack introns and contain a major transcription initiation site located 33-37 nucleotides downstream of a TATA box consensus sequence (Josefsson *et al.*, 1987; Scofield and Crouch, 1987).

Cruciferin and napin are detected only in the axis and cotyledons of maturing embryos, and therefore their expression is restricted to embryogenesis (Crouch and Sussex, 1981). Their deposition is not organ or tissue specific. However, it has recently been discovered that cruciferin and napin mRNA accumulate in the many different cell types at distinctly different times in different spatial distributions throughout the embryo (Fernandez *et al.*, 1991). The patterns of storage protein mRNA accumulation seem to reflect early events associated with the establishment of the apical meristems. The studies suggest that the total storage protein mRNA observed in Northern analysis is the sum of three distinct waves, each wave associated with mRNA accumulation in specific cell types (Fernandez *et al.*, 1991). The regulation of these waves is controlled in part by an internal clock that is set to respond to a spatial gradient of some factor situated at a critical point in time during early development.

The fact that both storage proteins are similar in their temporal and spatial regulation would suggest that the regulation of the genes is accomplished through similar controls and mechanisms. However, based on

the available sequence data, cruciferin and napin have little homology between each other (Crouch *et al.*, 1983; Simon *et al.*, 1985; Ericson *et al.*, 1986; Scofield and Crouch, 1987). Furthermore, cis-acting elements that regulate the expression of globulin like genes have been found in other systems such as the Leg box in *Vicia faba* (Baumlein *et al.*, 1986), *Glycine max* (Kitamura *et al.*, 1990), and *Pisum sativum* (Lycett *et al.*, 1985) have not been observed in the uptream regions of cruciferin.

Earlier investigations revealed no sequence homology to published cis-acting elements in the 5'-upstream region of the napin genes (Scofield and Crouch 1987), however, alternating purine-pyrimidine nucleotides were observed that have also been observed in a viral enhancer (Lusky *et al.*, 1983). Sequences related to AT-rich sequence, RY-repeats, and G-box like motifs are present, but remain to be characterized (Stalberg *et al.*, 1993). Furthermore, upstream analysis of napin has demonstrated nuclear factors binding promoter regions (Ericson *et al.*, 1986; Josefsson *et al.*, 1987), and deletion analysis has revealed important regions necessary for proper regulation (Stalberg *et al.*, 1993).

One line of evidence suggesting that cruciferin and napin expression is driven in a manner similar to other storage proteins is through the application of transgenic technology. Altenbach and co-workers (1992) observed a high level of expresssion and accumulation of a Brazil nut albumin in seeds of transgenic canola. In these experiments a chimeric contruct consisting of a phaseolin promoter linked to a methionine-rich Brazil nut albumin (Altenbach *et al.*, 1989) was expressed correctly both temporally and spatially in transgenic canola plants (Altenbach *et al.*, 1992). Furthermore, a napin promoter linked to a pea seed 2S albumin resulted in the expression of the message in transgenic tobacco plants at the proper developmental period in developing seed (Stayton *et al.*, 1991). Goldberg *et al.* (1989), after studying 5'deletion mutants of seed storage proteins in transgenic tobacco plants, concluded that the sequences necessary for temporal and tissue-specific expression lie in the 5' region

of the structural gene, and separate DNA elements in the 5' region are responsible for the control of the quantitative level and temporal/spatial regulation of these genes. The positioning of these elements differs between genes and species. These results taken together suggest that functional conservation of regulatory mechanisms exists between the seed storage protein promoters even though they display divergence in their promoter sequences.

Although the specifics of storage protein regulation have not been elucidated to date, one of the most likely mediators of regulation has been shown to be ABA. The application of exogenous ABA has been shown to promote the synthesis of storage protein messages and proteins in soybean (Bray and Beachy, 1985; Eisenberg and Mascarenhas, 1985), rice (Stinissen *et al.*, 1984), and wheat (Williamson *et al.*, 1985; Raikhel and Quatrano, 1986; Raihkel and Wilkins, 1987). These effects have also been demonstrated in *B. napus* (Crouch and Sussex, 1981; Crouch *et al.*, 1985; Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989; Wilen *et al.*, 1991). There is a strong correlation between endogenous ABA levels and cruciferin mRNA levels (Finkelstein *et al.*, 1985). Endogenous ABA levels peak at approximately 35 and 38 DPA (Finkelstein *et al.*, 1985). The same pattern of ABA levels in embryonic tissue has also been observed in *Phaseolus vulgaris* (Hsu, 1979), wheat (King, 1976), barley (Naumann and Dorffling, 1982), and in a related crucifer, *Arabidopsis* (Karssen *et al.*, 1983). In *Arabidopsis*, the initial peak is maternal in origin, while the secondary peak is derived from the embryonic tissue (Karssen *et al.*, 1983). Other effectors of storage protein synthesis include jasmonic acid (Anderson *et al.*, 1989; Mason and Mullet, 1990; Wilen *et al.*, 1991) and osmotica (Finkelstein and Crouch, 1986).

Although both cruciferin and napin are similar in their developmental and spatial expression they deviate slightly in their temporal expression. Cruciferin mRNA is detected at 23 DPA, accumulates to peak levels at 38 DPA (representing about 11% of the total embryo

mRNA), and thereafter declines to barely detectable levels in the mature dry seed (Finkelstein and Crouch, 1984; Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989). Napin transcription is detected at 18 DPA and increases to high levels until 33 DPA (representing about 8% of the total embryo mRNA), afterwhich the mRNA levels fall off (DeLisle and Crouch, 1989).

Cruciferin and napin are controlled differently at the transcriptional and post-transcriptional levels. After 38 DPA cruciferin mRNA steady state levels remain the same whereas the transcription rate decreases. Napin mRNA transcription, on the other hand, remains high, but the mRNA levels decline after 33 DPA (DeLisle and Crouch, 1989). Since mRNA levels are dependent upon both mRNA synthesis and degradation rates, it follows that cruciferin mRNA is more stable than napin mRNA. This phenomenon has also been observed for storage protein synthesis in other species including *Pisum sativum* (Evans *et al.*, 1984), *Phaseolus vulgaris* (Chappel and Chrispeels, 1986) and *Glycine max* (Walling *et al.*, 1986).

Based on immunoelectrophoresis, cruciferin is present at 25 DPA (0.26 µg/antigen/embryo), and accumulates at a rate of 2.5 µg/day until day 30. Maximum accumulation occurs between days 30 and 50 (26 µg/day) before decreasing to 3 µg/d during maturation (50-60 DPA) (Crouch and Sussex, 1981). Based on SDS-PAGE, Coomassie stained polypeptides corresponding to napin accumulated at the same rate as cruciferin until 42 DPA when accumulation stops (Crouch and Sussex, 1981).

Immunolocalization of cruciferin and napin has provided insight into the deposition of storage protein within the cell. Both cruciferin and napin are deposited together around the edges of protein bodies\vacuoles in young cotyledon cells (Murphy *et al.*, 1989b). Deposition continues centripedally, filling the protein body, and precipitating to form "globuloid" inclusions by 56 DPA (Gifford *et al.*, 1982; Murphy *et al.*, 1989b). In the mature seed, protein bodies are located within the center

of the cell (Kuras, 1984). This indicates that both cruciferin and napin are deposited together by the same mechanism. The mechanism is believed to involve the direct insertion of the newly synthesized proteins into the protein bodies derived from fragmentation of the central vacuole. The processing of the precursor forms into the mature oligomeric structures is believed to occur within the protein body or immediately prior to its insertion with the assistance of the RER and\or the Golgi apparatus (Murphy *et al.*, 1989b). In many seeds it has been demonstrated that storage proteins are initially synthesized on the RER in an unprocessed or precursor form (Larkins, 1981; Higgins, 1985; Bewley and Black, 1994) and transported intracellularly via vesicles through the Golgi apparatus (Greenwood and Chrispeels, 1985; Higgins, 1985; Fukasawa et al., 1988; Chrispeels, 1991; Nakamura and Matsuoka, 1993; Shewry *et al.*, 1995).

Immunocytochemical localization of cruciferin and napin throughout the embryo has also been studied in detail. Hoglund *et al.* (1992) observed the initiation of napin deposition at 20 DPA and cruciferin deposition at approximately 25 DPA. The accumulation patterns of the storage proteins progressed in a manner closely reflecting previous immunological studies (Crouch and Sussex, 1981; DeLisle and Crouch, 1989). Both proteins were detected initially at the tip of the axis with continued deposition occurring through the axis to the base of the outer cotyledon and towards its tip (Hoglund *et al.*, 1992). This was followed by deposition in the inner cotyledon. Little or no deposition was observed in myrosinase containing cells or procambium. This observation suggests that there might be some slight tissue specificity in accordance with the findings of storage mRNA accumulation studied by Fernandez and co-workers (1991).

3.4. Desiccation.

The final phase of embryogenesis is desiccation. During this period of development embryogenesis ceases as the seed begins to lose water until

a period of developmental arrest is attained (Finkelstein and Crouch, 1984). Embryo maturation is separated from post-germinative growth temporally by the process of desiccation, a physiological state of quiescence, and the processes of imbibition and subsequent germination (Bisgrove *et al.*, 1995).

In *B. napus*, excised zygotic embryos taken from the predesiccation stage (30-35 DPA) and late desiccation stage (45-50 DPA) will germinate precociously when cultured on basal media (Finkelstein and Crouch, 1984) by passing later stages of embryogeny (Crouch *et al.*,1985). Predesiccation embryos display morphological characteristics of germination (root growth and hypocotyl extension), however they also retain biochemical characteristics of embryogenic growth (cruciferin expression and accumulation). On the other hand, precociously germinated desiccation staged embryos gradually degrade storage protein over a period of several weeks, and exhibit normal seedling development. Exogenous application of ABA to excised embryos in the predesiccation phase leads to increased storage protein expression and accumulation, and inhibition of germination (Finkelstein *et al.*,1985). During the desiccation phase, similar concentrations of ABA applied to excised zygotic embryos did not result in increases in storage protein expression and accumulation (Finkelstein *et al.*, 1985). Osmotica produced similar results; however, it was observed that embryos cultured on 12.5% sorbitol maintain high cruciferin mRNA levels during the desiccation phase (Finkelstein and Crouch, 1986). This has led to the hypothesis that the maturation process is the result of two distinct stages (Finkelstein and Crouch, 1985; Finkelstein *et al.*, 1985; Finkelstein and Crouch, 1986). In this scenario, ABA is important during early embryogeny by regulating the expression of storage proteins genes and preventing precocious germination. The high levels of ABA also prevent the uptake of water during the deposition of solutes from the maternal tissue (Finkelstein and Crouch, 1985; Finkelstein *et al.*, 1985). During later stages of

embryogenesis the embryos become insensitive to ABA and desiccation then becomes the main factor preventing precocious germination. This is supported by the fact that desiccation is necessary for the developmental transition from embryogeny to developmental arrest.

These observations have been often cited to negate the idea that embryogenesis and germination programs are mutually exclusive and a simple switch controls their progression. However, in a follow up study using excised zygotic embryos at the same predesiccation and desiccation stages as Finkelstein and Crouch (1984), Bisgrove *et al.* (1995) observed that mRNAs characteristic of embryo maturation (N2: napin, C1: cruciferin; Crouch *et al.*, 1983, 1985; LEA76; Harada *et al.* 1989) were segregated from mRNAs characteristic of germination and post-germinative growth (COT1; CA25; CA8; AX92; Harada *et al.*, 1988). When messages were present simultaneously they were segregated by organ specificity with accumulation in the hypocotyl preceding accumulation in the cotyledons. Sets of developmental messages occurring in any given organ accumulated in a sequential pattern and not simultaneously. Furthermore, studies employing the 5'upstream regions of napin in transgenic tobacco revealed that desiccation down-regulated a reporter gene, suggesting that the regulation of napin may be controlled by processes occurring during desiccation (Jiang *et al.*, 1995). Therefore, the apparent overlap of developmental programs observed in previous studies (Finkelstein and Crouch, 1985; Finkelstein *et al.*, 1985; Finkelstein and Crouch, 1986) has been misleading, and the current results are in agreement with the hypothesis that embryo maturation and postgerminative growth are mutually exclusive, occurring sequentially, with developmental switches in place (Kermode, 1990).

In cotton, events during late embryogenesis have been further delineated into postabscission, predesiccation, desiccation and mature embryo stages (Galau *et al.*, 1991). By following molecular markers throughout these stages they concluded that excision of the ovule induces

the postabscission program and desiccation followed by imbibition then turns on the germination program (Hughes and Galau, 1991). In this scenario desiccation did not play any role in the control of gene expression, and, therefore, did not serve as the signal for switching from the embryogeny to germination programs. Rather, abscission is the criterion for the developmental switch, with excision-induced expression being enhanced by exogenous ABA application and precocious desiccation. When these experiments were performed on *B. napus* similar results were obtained (Jakobsen *et al.*, 1994). However, unpublished results (Dr. A.M. Johnson-Flanagan personal communication) implicated that abscission is not part of the developmental process in *Arabidopsis* seed. The observation of simultaneous induction of the postabscission and germination programs in excised *Brassica* embryos was believed to reflect stress reponses of the germinating embryos and not the effects of endogenous growth regulators (Jakobsen *et al.*, 1994). It has been proposed that a number of developmental processes or physiological conditions serve as the switch in turning the developmental programs from embryo maturation to germination and postgerminative growth. These include the growth regulator ABA, abscission of the embryo, and desiccation. The nature or mechanism of the switch is still under intense investigation. A number of techniques are currently being employed to generate mutants (Young and Phillips, 1994). The characterization of these mutants will provide information in delineating developmental processes and programming during embryogenesis. For instance, characterization of the *Arabidopsis raspberry* mutant revealed that histodifferentiation occured in the absence of normal morphogenic development, and early embryonic cells regulate their proper gene sets in the correct temporal and spatial context independent of normal morphogenic events (Yadegari *et al.*, 1994). These mutants and others arrested at the globular stage appear to be arrested only morphologically, and, therefore, some may possess altered genes involved in the biosynthesis and transport of auxin, which has been shown to play

a role in cotyledon initiation and hypocotyl elongation (Cooke and Cohen, 1993; Liu *et al.*, 1993). Another *Arabidopsis* mutant expresses postgerminative characteristics precociously during embryogenesis, and therefore, the normal gene product of the mutation is believed to play a role in regulating late embryogenesis (West *et al.*, 1994). Two other *Arabidopsis* mutants, *abi3* and *fus3*, also exhibit disruptions in developmental programming during late embryogenesis (Keith *et al.*, 1994; Parcy *et al.*, 1994). These and other studies suggest that during embryogenesis there are a number of concurrently operating regulatory pathways that are independent of each other. Each regulatory pathway would be contolled by different cis-acting DNA sequences and trans-acting factors under the direct or indirect control of different environmental cues and\or growth regulating factors. A more definitive model proposed by Thomas (1993) suggests developmental gene regulation is bipartite. Proximal promoter regions would specify seed-specific expression and more distal regions would refine and\or enhance the expression patterns conferred by the proximal regions (Thomas, 1993). In addition, other elements would maintain hierarchical control over the distal elements.

3.5. The Mature Embryo.

There are two separate classes of mRNAs within the mature rape embryo: residual mRNAs and conserved or stored mRNAs (Kermode, 1990). Residual mRNAs are produced during seed development and persist through late maturation and desiccation. These messages are not necessary to the germination process and may be degraded quickly upon imbibition. Conserved or stored messages are produced during seed development and are available for translation upon hydration. These messages are an integral part of germination and can be further subcategorized into mRNAs for (a) essential enzymes required for intermediary metabolism not unique to germination, and (b) proteins necessary for successful germination (these will be dealt with in the next section).

During the maturation phase, a group of residual mRNAs derived from the *Lea* genes (Galau *et al.*, 1986) increase. In *Brassica napus* these mRNAs increase dramatically in developing seeds (26-35 DAF) persist at high levels when the embryos begin to desiccate (38-42 DAF), remain at high levels in the dry seed (60 DAF), and decline rapidly upon imbibition during germination (absent after 16-24 HAI) (Harada *et al.*, 1989). This species of message has been observed in cotton (Baker *et al.*, 1988), carrot (Choi *et al.*, 1987), barley (Hong *et al.*, 1988), and rice (Mundy and Chua, 1988).

From the deduced amino acid sequences the highly hydrophilic LEA proteins have been grouped into three classes (Dure *et al.*, 1989). LEA76, cloned from *B. napus*, (Harada *et al.*, 1989) belongs to class 3 LEAs and is characterized as containing a repeating tract of 11 amino acids that possibly exists in an alpha-helix with twisting amphiphilic surfaces (Dure *et al.*, 1989). Another LEA message, B86, cloned from a related crucifer, radish, belongs to class 1 LEAs and hybridizes to a 850 bp message in the *B. napus* (Raynal *et al.*, 1989). Although the function of LEA proteins has not been empirically demonstrated they are believed to serve as desiccation protectants.

There is also a small group of conserved or stored mRNAs activated during late embryogenesis that are induced during germination and seedling development (Harada *et al.*, 1988; Goldberg *et al.*, 1989). In *B. napus* members of this class were expressed in a spatial manner related to the cotyledons and axis. One clone, pCOT44 was present in low amounts during late embryogeny, increased during germination (first 24 HAI), and remained high during postgermination in the cotyledons (Harada *et al.*, 1988; Dietrich *et al.*, 1989). The deduced amino acid sequence of COT 44 is homologous to cysteine proteinases (Dietrich *et al.*, 1989). Conversely another clone, pAX92, was expressed in a similar manner temporally, but exhibited higher expression in the axis of germinating seedlings (Harada *et al.*, 1988; Dietrich *et al.*, 1989).

These events of seed development culminate in the formation of a mature seed packed with storage products throughout the cotyledons and axis (Kuras, 1984). The mature embryo cells also possess nearly all the organelles present in active cells except Golgi bodies (Kuras, 1984). All cellular constituents are densely packed as a consequence of the low hydration level. The proximity of the organelles to each other allows for the basic metabolic processes to occur during quiescence. The cells with the highest degree of similarity to that of active cells are those of the columella of the root cap (Kuras, 1984). The columella are the cells that give rise to the root cap. This is not surprising because these cells are the first to enter the external environment after extension of the radicle during germination, and are the first to provide nutrients, water and minerals to the seedling during emergence.

4.0. Germination.

Desiccation marks the end of embryo maturation and completes the embryogenesis program. *B. napus* seeds do not enter true dormancy but remain in a state of quiescence, and therefore will enter germination with the appropriate environmental conditions. In many annuals, such as *B. napus*, dormancy has been bred out to acquire more uniform germination (Adler *et al.*, 1993 and references therein).

Germination is the developmental period that begins with water uptake and ends with radicle emergence through the seed coat (Bewley and Black, 1994). Prior to imbibition, canola seed have a very low hydration level and low metabolic activity (Kuras, 1984). Canola seed requires 60-75% relative moisture content between the temperatures of 2-25°C, in the presence of oxygen, to germinate (Canola Growers Manual, 1991). Often the early stages of germination are reversible, i.e. seeds that are partially hydrated can been lyophilized and are still viable when hydrated again. This process can be repeated until a later stage when the seed is committed to continuing the process of germination (Mayer and Marbach,

1981). Thereafter, drying down the seed will result in the death of the seed.

The process of germination is subdivided into three phases (Tissaoui and Come, 1975; Simon, 1984; Vertucci, 1989; Bewley and Black, 1994). During phase I, rapid hydration (imbibition) of the seed occurs and metabolism begins. During phase II, a lag follows while major metabolic events take place in preparation for radicle emergence. The final event, phase III, is characterized by radicle elongation, and marks the end of germination and the beginning of seedling growth.

4.1. Phases of Germination.

Mature canola seeds have a low moisture content, generally less than 10%, and therefore are considered "orthodox" seed (Bewley and Black, 1994) as opposed to "unorthodox" or recalcitrant seed. The water potential of a seed is a combination of three components: the osmotic potential, the matrix component, and the pressure potential (Bewley and Black, 1994). Oil seeds generally have a lower water content relative to species with high starch content (Simon, 1984), and therefore have a negative matrix component (<0) and a highly negative osmotic potential (<0). In addition, small seeds, such as *B. napus*, produce mucilage in the seed coat and are relatively smooth with a high surface area/volume ratio, resulting in a slightly positive pressure potential (>0). Therefore, in *B. napus* the first phase of imbibition proceeds rapidly because of the low water potential of the seed (on the order of -100 MPa) (Shaykewich and Williams, 1971; Shaykewich, 1973). As the uptake of water increases the force of water from the external environment into the seed decreases logarithmically (Shaykewich and Williams, 1971; Shaykewich, 1973; Bewley and Black, 1994). The seed coat affords some protection during this time as it slows the rate of imbibition (Simon, 1984). As the seed takes up water the gradient diminishes until the uptake of water slows and enters a lag period.

Respiration begins rapidly after imbibition commences and increases as the water potential rises in the seed (Simon, 1984). The progression of respiration is also triphasic, increasing rapidly upon imbibition, plateauing after a variable duration, then increasing again (Simon, 1984; Bewley and Black, 1994). The initial rise is associated with hydration of the seed and activation of the mitochondrial enzymes (Mayer and Poljakoff-Mayber, 1989; Bewley and Black, 1994). The second increase in respiration is associated with cell division during the third phase of germination (Simon, 1984).

Directly related to the increase in respiration, is the increase in energy charge as defined by Atkinson (1977). ATP production increases rapidly through both substrate level phosphorylation followed later by oxidative phosphorylation, providing energy to the metabolic machinery necessary to support germination (Mayer and Marbach, 1981; Simon, 1984). In a closely related crucifer, *Sinapsis alba*, tritium from tritiated water was incorporated rapidly into oxo-acid intermediates of the Krebs cycle (Spedding and Wilson, 1968). It was concluded that incorporation was the result of amino acid metabolism, probably deaminations followed by transaminations. The researchers postulated that metabolism of the Krebs cycle organic acids occurred shortly after the initial amino acid metabolism (30 min) either in the Krebs or glyoxylate cycle to produce the necessary energy needed for the rapid changes occurring in the embryo. Furthermore, the energy-producing cycles are supplied later (3 hrs) with acetyl-CoA necessary for the degradation of the lipid reserves. In *B. napus*, starch grains appear within 3 hours of imbibition in the columella of the root cap (Kuras, 1986). This indicates a very early initial enzymatic transformation of storage lipids into carbohydrates. Furthermore, starch is observed to accumulate in the embryonic cells just prior to germination (Kuras, 1986), providing the energy for intensified respiration during germination. These starch grains disappear soon after germination (Kuras, 1986).

Protein synthesis also commences at the start of imbibition, producing proteins, such as enzymes of glycolysis, that are essential to intermediary metabolism (Bewley and Black, 1994). Shortly after the loss of residual mRNA during imbibition (Kermode, 1990), new messages are produced, some that encode novel proteins involved in the mobilization of storage reserves after germination (Simon, 1984; Sanchez-Martinez *et al.*, 1986; Lane, 1991; Bewley and Black, 1994). In rape seeds, RNA synthesis is detected in the outermost cells of the embryo two hours after imbibition, followed by synthesis in other cells (Payne *et al.*, 1978). DNA synthesis (endoreduplication) lags behind RNA and protein synthesis, just prior to the start of cell division and after radicle elongation (Mayer and Marbach, 1981; Simon, 1984; Kuras, 1986; Bewley and Black, 1994). The speed of these events all play a role in controlling the length of the lag phase. These events may be delayed by factors such as inadequate product accumulation, lack of cell preparation (organellar synthesis and/or maturation, ie. mitochondria and glyoxysomes), or lack of structural reorganization (cell wall loosening) all of which are necessary for radicle extension.

Only germinating seeds enter phase III (Bewley and Black, 1994), which is concurrent with radicle protrusion through the seed coat. Initially, the radicle elongates slowly by cell expansion, followed by rapid growth and cell division. Eventually the radicle breaks through the testa to end germination (Esau, 1977; Kuras, 1986; Kuras, 1987; Bewley and Black, 1994). After germination, water uptake increases and the storage reserves are utilized to support seedling growth until photosynthesis and autotrophic growth can be maintained.

4.2. Low Temperature Effects During Germination.

Intrinsic factors influencing the success of germination include mucilage content (Eskin, 1992), testa thickness (Teran *et al.*, 1994), genotype (Wilson *et al.*, 1992), seedlot (Perry, 1972; Barber *et al.*,

1991), storage conditions (Frisbee *et al.*, 1988; Bernal-Lugo and Leopold, 1992; Filho and Ellis, 1992; Vertucci, 1992), seed age (Dawidowicz-Grzegorzewska and Podstolski, 1992), seed pre-treatments (Nelson *et al.*, 1984; Amritphale *et al.*, 1989; Basra *et al.*, 1989; Coolbear and McGill, 1990; Omari, 1992; Todari and Negi, 1992), seed injury (Buntin *et al.*, 1995), and conditions under which the seed matures (Gray and Thomas, 1982; Taylor *et al.*, 1991). The environment also has a very large influence on the rate and success of germination.

Generally, temperatures below the optimum result in progressively poorer germination (Liptay and Schopfer, 1983; Liengsiri and Hellum, 1988; Mohapatra and Suggs, 1989; Steiner and Jacobsen, 1992; Leviatov *et al.*, 1994; O'Conner and Gusta, 1994). Low temperature slows the rate of imbibition (Vertucci, 1989), and may damage embryos, preventing germination (Bramlage *et al.*, 1979; Bradbeer, 1988; Woodstock, 1988). This in turn may result in slow, asynchronous germination, leading to poor stand establishment (Stewart *et al.*, 1990; Barber *et al.*, 1991), and ultimately, reduced yield (Phillips and Youngman, 1971; Hobbs and Obendorf, 1972).

In tobacco, cucumber, and mustard, low temperature also increases the period of the lag during phase II (Simon *et al.*, 1976). In addition, low temperature results in reduced radicle growth and lower respiration rates. Failure to germinate at low temperature may be the result of an inability to complete phase II or an inablility to enter phase III of germination because of the denaturation of proteins (Simon *et al.*, 1976). Another possible explanation for the failure to germinate, is that the cellular membranes of the seed are disrupted and are unable to reconstitute properly at low temperature, resulting in the leakage of cellular components and restricting compartmentation of the organelles (Mayer and Marbach, 1981 and references therein; Bewley and Black, 1994).

In *B. napus*, low temperature germination studies have been limited to assessing germination rates under low temperature regimes (Acharya *et*

al., 1983; Kondra *et al.*, 1983; Livingstone and deJong, 1990; Barber *et al.*, 1991; Mills, 1993). As with other species (Mohaptra and Suggs, 1989; Addae and Pearson, 1992), *Brassica* genotypes (cultivars) display differences in germination rates at low temperatures (Acharya *et al.*, 1983; Wilson *et al.*, 1992; Mills, 1993). Acharya *et al.* (1983) determined that 10°C was the best temperature to select for superior low temperature germination in Westar; however, selection for rapid germination at this temperature produced inconsistent results. Similarily, King *et al.* (1986) found that such selections did not necessarily lead to improved performance. These results suggest complex control of germination and early seedling growth.

In tomatoes, cutting the seed coat may increase germination percentage and rate at low temperature (Singer *et al.*, 1989). While, this has not been assessed in *B. napus*, seed priming has been shown to result in more uniform and rapid low temperature germination (Rao and Dao, 1987; Zheng *et al.*, 1994). Priming with PEG increased the synchronization and rate of germination at low temperature in wheat, oats (Akelehiywot and Bewley, 1977), soybean (Knypl and Khan, 1981), parsley (Akers *et al.*, 1987), maize (Basra *et al.*, 1988), carrot, celery and onion (Szafirowska *et al.*, 1981; Brocklehurst and Dearman, 1983a, 1983b). In four seedlots of leek, priming improved the rate and synchronicity of seed germination in the slower germinating lots (Brocklehurst and Dearman, 1984). In all of these studies, the hydration level of the seed is raised under controlled conditions, followed by dehydrating the seed to original moisture levels (Heydecker and Gibbons, 1978; Heydecker and Coolbear, 1979; Brocklehurst and Dearman, 1984; Fujikura *et al.*, 1993).

Primed seeds have enhanced metabolic activity (respiration rates) (Fu *et al.*, 1987; Yan, 1987), enzymes involved in the mobilization of seed reserves are activated (Khan *et al.*, 1978), there is improved synthesis of RNA and protein (Khan *et al.*, 1978; Coolbear and Grierson, 1979; Knypl *et al.*, 1980; Fu *et al.*, 1987) and membrane integrity is restored, repaired,

or synthesized (Burgess and Powell, 1984; Fu *et al.*, 1987; Basra *et al.*, 1988). Priming in *B. napus* is believed to improve germination by initiating metabolic events and/or the leaching of germination inhibitors (Zheng *et al.*, 1994). The presence of a water soluble germination inhibitor in *B. napus*, modulated by low temperature and light has been postulated (Bazanska and Lewak, 1986). Additionally, the inhibitory effects of ABA (Schopfer and Plachy, 1984; Schopfer and Plachy, 1985; Sharma *et al.*, 1992) and phenolics (Nandakumar and Rangaswamy, 1985) on *B. napus* germination have also been demonstrated. These studies are interesting, but fail to demonstrate any of the underlying biochemical or molecular processes occurring during low temperature germination.

5.0. Early Seedling Growth.

Whereas maturing embryos increase in size through both cellular division and expansion throughout the embryo, germinating seedlings grow by cellular division followed by subsequent expansion basipetally in the localized regions of the meristems (Finkelstein and Crouch, 1984). Seedling growth in *B. napus* proceeds in an epigeal fashion, in which the cotyledons are raised by expansion and growth in the hypocotyl (Bewley and Black, 1994). During this period the stored reserves of the embryo are utilized by the seedling to support growth until autotrophic growth can be maintained.

5.1. Storage Reserve Mobilization.

5.1.A. Lipid Mobilization.

Germinating oil seeds rapidly mobilize storage lipids (Doman *et al.*, 1982; Murphy *et al.*, 1989a; Qouta *et al.*, 1991) and convert them to sucrose, the primary nutrient during heterotrophic growth (Beevers, 1979; Trelease, 1984). In *B. napus*, total lipids steadily decrease between day 1 through to day 10 in germinating seedlings (Lin and Huang, 1983). Prior to this conversion, enzyme synthesis and organelle assembly is required to

produce glyoxylate cycle enzymes, such as ICL and MS, and the glyoxysomes where these enzymes are localized (Breidenbach and Beevers, 1967; Gerhardt and Beevers, 1970).

The cells in the mature *B. napus* seed are packed with oil bodies (Kuras, 1984). The conversion of lipids to sucrose involves the oil bodies themselves, glyoxysomes, mitochondria, and the cytosol (Trelease and Doman, 1984; Bewley and Black, 1994). Associated with the oil bodies are lipases (Lin and Huang, 1983), which convert the TAGs to FFAs. The FFAs then enter the glyoxysome, and via β-oxidation result in the formation of acetyl-CoA (Lin and Huang, 1983; Mathews and van Holde, 1990). The high energy form of acetate (acetyl-CoA) then enters the glyoxylate cycle to eventually result in the formation of succinate (Kornberg and Beevers, 1957; Breidenbach *et al.*, 1967). Succinate is converted to malate in the mitochondria (Beevers, 1982) via the Krebs cycle, and finally converted to sucrose through gluconeogenesis in the cytosol (Mathews and van Holde, 1990).

5.1.A.a. Lipases.

The lipids contained within the oil bodies are hydrolyzed to glycerol and fatty acids by lipase(s) (glycerolester hydrolase, EC 3.1.1.3) (Huang, 1992). In *Brassica*, lipase activity is absent in the mature seed, and increases during germination and postgerminative growth (4 day old seedlings), concomitant with decreases in oil reserves (Theimer and Rosnitschek, 1978; Lin and Huang, 1983; Hills and Murphy, 1988; Huang, 1992). Although the factors involved in initiating lipase induction are unknown, gibberellins have been shown to increase lipase activity while abscisic acid reduces activity in germinated *B. napus* seeds (Imeson *et al.*, 1993). Lipase inhibitors, present in the 2-4S subcellular protein fraction, were found to decrease in soybean cotyledons after germination (Wang and Huang, 1984). Furthermore, the inhibitor reduced lipase activity in rape and mustard; however, the presence of lipase inhibitors

is likely not a factor in determining the rate of lypolysis in rape, as lipase activity is high.

In *B. napus*, two lipases are expressed during lipid mobilization. One lipase is loosely associated with the oil bodies, has a pH optimum around 6.5, and is most active towards TAGs (Lin and Huang, 1983). Presumably the lipase attaches to the membrane of oil bodies after its synthesis (Huang, 1992). The recognition site of the lipase is unknown, but oleosins have been suggested to serve in this capacity (Huang *et al.*, 1987; Lee and Huang, 1991). Another lipase is found in the microsomal fraction (Theimer and Rosnitschek, 1978; Hills and Murphy, 1988) and displays a pH optimum between 7.5 and 9.0. Both lipases correspond to a 56 kD protein detected in both the oil body and microsomal fractions (Murphy *et al.*, 1989a). There is some debate in the literature whether or not the microsomal lipase represents a newly synthesized enzyme or represents the remnants of degraded oil bodies (Murphy *et al.*, 1989a; Huang, 1992).

A long chain acyl-CoA synthase (E.C. 6.2.1.3) has been detected in *B. napus* dry seeds. It increases during imbibition and declines after two days of germination (Olsen and Lusk, 1994). This enzyme was recovered from the lipid body membrane preparations, requires cofactors including ATP, and is believed to play a role in the processing of fatty acids stored in oil bodies. This is further supported by the detection of an ATPase associated with oil body membranes that displays maximal activity concurrently with lipase activity (Olsen and Weatherman, 1992).

During the course of postgerminative growth in *B. napus*, the storage cotyledons synthesize chlorophyll and carry out photosynthesis after the depletion of the storage reserves (Huang, 1992). After the TAGs have been depleted, "ghosts" of the oil bodies remain in the cytosol (Wanner and Theimer, 1978) and their fate remains unknown (Huang, 1992).

5.1.A.b. Glyoxysomes.

Glyoxysomes were first isolated from castor bean endosperm (Breidenbach *et al.*, 1967; Breidenbach and Beevers, 1967; Cooper and Beevers, 1969). Later it was observed that the protein content from isolated glyoxysomes and glyoxysome specific enzyme activity (MS and ICL) increased over the first 4 to 5 days of germination (Gerhardt and Beevers, 1970). Since that time there has been an intense study of glyoxysome biogenesis and characterization of the ICL and MS enzymes.

Peroxisomes are dense (1.25 g/cm^3) non-autonomous (do not self replicate) single membrane bounded organelles containing catalase and H_2O_2-producing oxidases. Their matrix is finely granular, sometimes containing paracrystalline or dense amorphous inclusions (Trelease, 1984; Olsen and Harada, 1995; Bewley and Black, 1994). Glyoxysomes represent a specialized group of peroxisomes found during postgerminative growth in oilseed plants (Trelease, 1984), but have also been observed in senescent organs (cotyledons, leaves and petals) (Olsen *et al.*, 1993, Zhang *et al.*, 1994; Olsen and Harada, 1995). In addition to the aformentioned characteristics, glyoxysomes also possess the β-oxidation enzymes and glyoxylate cycle enzymes (Trelease, 1984; Vanni *et al.*, 1990).

The appearance of glyoxysomes increases dramatically after germination (Gerhardt and Beevers, 1970). The formation of glyoxysomes has always been an area of intense debate leading to the proposal of different models (see Trelease, 1984; Chapman and Trelease, 1991). Currently, peroxisomes are believed to originate by growth and division of pre-existing peroxisomes (Olsen and Harada, 1995), and not from budding of the ER (Trelease, 1984). Growth of the glyoxysomes is accomplished through post-translational import of proteins and the addition of lipids from other sources (Chapman and Trelease, 1991; Keller *et al.*, 1991; Olsen and Harada, 1995). If this theory is correct, then the non-autonomous glyoxysomes in the germinating seed are derived from the gamete(s) in the same way as mitochondria and chloroplasts. Two markers of glyoxysomal function, ICL and MS, have been demonstrated to be

transcriptionally active in *Brassica* pollen (Zhang *et al.*, 1994). In addition, microbodies are present in the vegetative cell of mature pollen (Charzynska *et al.*, 1989). While this supports the hypothesis of parental inheritance, it raises serious questions as to the evolution of peroxisomes. Unfortuneatly, there is no mention of microbodies in the egg cell within the current literature, although concievably they could be present via the germ cells from which they are derived from. Regardless of their origins further research is required to assign a direct link between the presence of microbodies and their transformation into glyoxysomes.

5.1.A.c. Glyoxylate Cycle Enzymes: Isocitrate Lyase and Malate Synthase.

Isocitrate lyase (threo-D_s-isocitrate glyoxylate-lyase, E.C. 4.1.3.1) is a glyoxylate cycle enzyme responsible for converting isocitrate to glyoxylate and succinate (Vanni *et al.*, 1990). This reaction occurs at a branch point diverting isocitrate into the carbon conserving glyoxylate cycle, enabling the synthesis of one mole of succinate from two moles of acetate (as acety-CoA) (Vanni *et al.*, 1990). Isocitrate lyase is suspected to be one of the regulatory enzymes in the mobilization of lipid reserves (Malhotra *et al.*, 1984; Vanni and references therein, 1990) because the substrate isocitrate is partitioned between two competing cycles (Glyoxylate cycle and Krebs tricarboxylic acid cycle).

After isocitrate is cleaved to form glyoxylate and succinate, the glyoxylate re-enters the cycle, accepting another acetate molecule (acetyl-CoA) from β-oxidation. Malate synthase (L-malate glyoxylate-lyase [CoA-acetylating], E.C. 4.1.3.2) is the enzyme responsible for condensing the glyoxylate molecule with acetyl-CoA to form malate (Trelease and Doman, 1984; Mathews and van Holde, 1990; Bewley and Black, 1994).

ICL and MS have historically been used as biochemical markers of glyoxysomal function (Beevers, 1979; Breidenbach *et al.*, 1967; Gerhardt and Beevers, 1970; Trelease, 1984), but like glyoxysomes their expression

is not restricted to only germination and post-germinative growth; ICL is detected late in embryogenesis, in senescing organs, and pollen (Zhang *et al.*, 1994). The presence of ICL and MS transcripts during late embryogeny is puzzling. Both ICL and MS activity is detectable during late embryogeny and in the mature seed of *B. napus* (Ettinger and Harada, 1990). This suggests that both enzymes are functional during embryo maturation, but the mobilization of lipid reserves is prevented. Therefore, an inhibitor may be present that can block ICL activity and/or biochemical steps prior to the evolution of the substrates in the glyoxylate cycle. Extracts from ripening seeds have been shown to strongly inhibit ICL activity (Allen *et al.*, 1988). However, this is not believed to be the case in *Brassica* (Ettinger and Harada, 1990), rather gene activation may occur in response to distinct physiological signals or metabolic factors at different developmental stages (embryogenesis versus postgermination) (Comai *et al.*, 1992; Zhang *et al.*, 1993). An alternative hypothesis suggests genes may be regulated by a common mechanism at each stage of development, but distinct regulatory programs are in operation that either activate or repress specific genes.

ICL and MS transcripts are initially detected during late embryogeny 35-40 DPA (Comai *et al.*, 1989b). Upon imbibition, the transcripts increase dramatically and remain high over the first six days of imbibition (Comai *et al.*, 1989b). Thereafter, the messages decline and are no longer detected in non-senescing organs or leaves (Comai *et al.*, 1989b; Ettinger and Harada, 1990; Comai *et al.*, 1992). In *B. napus*, the accumulation of ICL and MS mRNA is transcriptionally controlled, but post-transcriptional processes lead to quantitative differences (Comai *et al.*, 1989b). Both transcripts display qualitatively similar temporal and spatial distributions, and both accumulate more in the cotyledons in comparison to the axis (Comai *et al.*, 1989b; Deitrich *et al.*, 1989).

ICL is encoded by at least six genes, and based upon divergences in the 5' and 3' untranslated regions, represents two subfamilies (Zhang *et*

al., 1993). mRNAs from both subfamilies accumulate during late maturation and during postgermination (Zhang *et al.*, 1993). Expression of the ICL genes results in at least three classes of mRNA (2.3 kb) during late embryogenesis and postgerminative growth, and therefore ICL accumulation is not believed to result from alternative expression of distinct members of the gene family (Zhang *et al.*, 1993).

Malate synthase is encoded by a small gene family (Comai *et al.*, 1989a) consisting of at least four classes (Comai *et al.*, 1992). Each class recognizes a 2.1 kb mRNA transcript, but the temporal distribution of each class differs with respect to developmental stage (late embryogenesis versus postgermination) (Comai *et al.*, 1992). Furthermore, one class (MS-C), either does not encode functional proteins or only contains parts of the genes (based on its genomic length) (Comai *et al.*, 1992).

ICL and MS are synthesized on free polysomes and are transferred post-translationally into glyoxysomes during biogenesis (Olsen and Harada, 1995) and/or following detachment of the vesicles from ER (Trelease, 1984; Vanni *et al.*, 1990). Tri-peptide consensus motifs, SRM and SRL, located at the carboxyl terminus of ICL and MS, respectively, target these enzymes to the glyoxysomes (Olsen *et al.*, 1993). These results suggest that functional glyoxysomes arise as a result of the synthesis of the glyoxysomal proteins, and, therefore, peroxisomal classes are most likely determined by gene regulation and not protein import (Olsen *et al.*, 1993).

Translation of the ICL and MS transcripts results in the formation of 62 kDa and 60 kDa subunits, which in turn are processed to the mature tetrameric (257 kDa) and octameric (510 kDa) proteins that are reponsible for the enzyme activities of ICL and MS *in vivo*, respectively (Ettinger and Harada, 1990). Both enzymes are active in the mature seed, and upon imbibition increase dramatically over the first 3-4 days. Thereafter, activity declines and is no longer detected in young leaves (Ettinger and Harada, 1990). The loss in activity is associated with the transition of

glyoxysomes into leaf-like peroxisomes, which lack the glyoxylate cycle enzymes (Zhang *et al.*, 1993 and references therein). Again the qualitative patterns of accumulation are similar with respect to temporal and spatial distributions, but differences in mRNA to protein levels between ICL and MS suggest that translational or post-translational processes are involved resulting in the differential accumulation of the proteins (Ettinger and Harada, 1990). This result is further supported by the loss in enzyme activity later during imbibition (after 4-6 DAI), without a proportionate decrease in proteins (Ettinger and Harada, 1990).

During phototropic transformation of the cotyledons, the mobilization of reserves is promoted by light; cotyledons grown in the light exhibit rapid removal of the stored materials in comparison to dark grown cotyledons (Allen *et al.*, 1988) in cotton. This allows the conservation of nutrients in the cotyledons until they are exposed to light. The same may also be true for *B. napus*, but this has not been assessed to date.

5.1.B. Storage Protein Mobilization.

According to Mikola (1983) the proteolysis of storage proteins occurs in three stages: (1) firstly, hydrolysis provides amino acids for the synthesis of hydrolytic enzymes required to breakdown the stored reserves (2) secondly, hydrolysis of the storage proteins provides amino acids to the growing seedling (3) and lastly, the mobilization system itself is broken down to provide the last ration of amino acids, just prior to the onset of autotrophic growth.

The breakdown of the stored nitrogen reserves is achieved by two main sub-groups of peptide hydrolases, endopeptidases (E.C. 3.4.21-E.C. 3.4.24.) and exopeptidases (E.C. 3.4.11-E.C. 3.4.17) (Dalling and Bhalla, 1984; Callis, 1995). Endopeptidases are further subdivided into other classes, and carry out cleavages at particular amino acid(s) within polypeptides. The exopeptidases are also further subdivided into six

major classes, and carry out cleavages of one or two amino acids from the ends of polypeptides. Those enzymes that cleave at the amino-terminus are referred to as aminopeptidases; those that cleave at the carboxy-terminus are called carboxypeptidases (Bewley and Black, 1994). Relatively few of these enzymes have been characterized. One previously mentioned putative peptidase, COT 44 (Dietrich *et al.*, 1989), has homology to a cysteine proteinase, and therefore may serve as a endopeptidase after germination. A leucine aminopeptidase (exopeptidase) from *Arabidopsis* has been cloned (Bartling and Weiler, 1992), and may provide some insight into protein mobilization during this period.

Once the amino acids have been liberated from the storage proteins, they are generally converted to asparagine and glutamine, and are translocated to areas of active growth (Bewley and Black, 1994). In *B. napus*, the breakdown of protein bodies occurs first in the radicle, then in the hypocotyl, and finally in the cotyledons (Kuras, 1987; Hoglund *et al.*, 1992). In *B. campestris*, the initiation of protein body digestion in different tissues is suggested to be triggered simply by imbibition (Bhandari and Chitralekha, 1984).

In *B. napus*, mature seed germinated in culture degraded cruciferin within one week (Finkelstein and Crouch, 1984). One mechanism is believed to be involved in the mobilization of cruciferin and napin (Murphy *et al.*, 1989b). In immunocytochemical studies, rapid mobilization of storage proteins occurs within the protein bodies two days after germination (Murphy *et al.*, 1989b; Hoglund *et al.*, 1992). Western blotting shows the breakdown continues 2-3 days after germination, with only small inclusions of both cruciferin and napin remaining (Murphy *et al.*, 1989b). By six days after germination both cruciferin and napin could no longer be detected (Murphy *et al.*, 1989b). After the mobilization of the storage proteins is completed, the remnants of the protein bodies fuse to form the central vacuole (Murphy *et al.*, 1989b; Hoglund *et al.*, 1992).

In *B. oleracea*, the mobilization of proteins precedes the breakdown

of lipids (Qouta *et al.*, 1991). This probably involves the *de novo* synthesis of enzymes required for the breakdown of the lipid reserves. Murphy *et al.* (1989b) also observed the breakdown of storage proteins (3 days after germination) preceding that of lipid bodies in *B. napus*. Oleosins, which represent up to 10% of the total protein in the mature seed, rapidly disappear concommitant with lipid depletion (Huang, 1992).

6.0. Artificial Embryogenesis.

6.1. Historical Perspective.

The use of tissue culture has developed over the years from humble beginnings. Hannig (1904) from Germany was the first to successfully culture excised embryos from crucifers. In the 1920's Dr. Lewis Knudson (1922) devised a formula (Knudson "C" formula) to combine plant nutrients with sugar, as a carbohydrate source, and agar. These mixtures are still available today, and are used to germinate sterile seedlings and propagate whole plants from tissue. The advent of callus culture in 1939 (Gautheret, 1939; Nobecourt, 1939; White, 1939) was soon followed by the induction of organogenesis from callus into shoots and roots with the use of plant growth hormones by Skoog and Tsuii (1948). Later, techniques were devised to propagate whole plants from single cells (protoplast culture) (Muir *et al.*, 1954; Miller *et al*, 1955; Skoog and Miller, 1957), from pith of tobacco, soybean, carrot and other dicots (Murashige and Skoog, 1962; Gray and Purohit, 1991) , from cell suspension cultures (Steward, 1958; Zimmerman, 1993 and references therein), and from anthers and microspores (Guha and Maheshwari, 1964).

Since its inception, tissue culture techniques (Wetter and Constabel, 1982; Kyte, 1987; Pierik, 1987) have been utilized by both commerce and the scientific community as a productive means of propagating plants and plant organs. It is now possible to propagate millions of identical plants (clones) from one small piece of plant tissue (explant)

(Pruski, 1994). In theory, any plant can be propagated by tissue culture because many plant cells are totipotent (Goldberg, 1988; Goldberg *et al.*, 1989). That is, some plant cells have the ability to regenerate complete plants, suggesting that under the proper conditions plant cells possess a complete gene set for all stages of differentiated growth (Goldberg, 1988).

In the current study, haploid embryos produced from microspores are employed to study the accumulation of storage proteins during embryogenesis. For these reasons the review of artificial embryogenesis will be limited to MDEs in *B. napus*.

6.2. Microspore-derived Embryos.

The processes involved in microsporogenesis have been well defined in angiosperms (Shivanna and Johri, 1975; Fabijanski *et al.*, 1992). Haploid embryo production is achieved through the isolation of microspores from the pollen sac at the uninucleate stage (at the first pollen mitosis prior to the formation of a binucleate microspore) (Fan *et al.*, 1988). Sterile isolation followed by *in vitro* culture leads to the formation of haploid embryos. Embryogenesis is induced by a short exposure to heat (Telmer *et al.*, 1993; Binarova *et al.*, 1994; Custers *et al.*, 1994). In *B. napus* cv. Topas, tissue culture techniques have been developed to produce large quantities of embryos (Lichter, 1982; Orr *et al.*, 1990; Takahata *et al.*, 1991; Lo and Pauls, 1992; Telmer *et al.*, 1992; Hansen and Svinnset, 1993; Iqbal *et al.*, 1994). These advances have enabled researchers to compare MDE to the zygotic embryos in biochemical and molecular terms.

6.3. Morphological, Biochemical, and Molecular Aspects.

Microspores, undergoing embryogenesis in culture, divide and grow in very similar patterns to those observed in zygotic embryos (Wilen, 1992). Embryogenesis has been reported to proceed through the same morphological stages (globular, heart, torpedo, and cotyledonary) displayed during

normal zygotic embryogenesis (Wilen, 1992; Yoon *et al.*, 1993). A few exceptions have been reported including the timing of the transitions in embryo growth and the overall size of the MDE at the cotyledonary stage (Wilen, 1992).

MDEs mimic the zygotic system in relation to lipid biosynthesis and accumulation (Taylor *et al.*, 1990; Chen and Beversdorf, 1991; Pomeroy *et al.*, 1991; Taylor *et al.*, 1992). Further, it has been shown that oilbody-associated proteins (oleosins) accumulate similarly to that in zygotic embryos (Wilen, 1992). MDEs also accumulate glucosinolates when cultured under proper conditions (McClellan *et al.*, 1993). Cruciferin and napin gene expression have been reported to be similar in zygotic and MDEs (Wilen, 1992), however, the MDEs do not accumulate these gene end products (Taylor *et al.*, 1990; Wilen, 1992), nor has the temporal development been studied. However, in a recent study Kennedy (1993) observed that under certian culture conditions in the presence of light, MDEs did accumulate protein bands corresponding to the molecular weights of cruciferin and napin. Therefore, by using these same culture conditions and confirming the presence of cruciferin and napin via western anaylsis, this same sytem could be utilized to study storage protein accumulation in MDEs.

MDEs are an ideal system to apply external factors as maternal influences are removed. Studies of exogenously applied ABA demonstrate accumulation of both cruciferin and napin transcripts (Wilen *et al.*, 1990; Wilen, 1992). ABA application to excised zygotic embryos in *B. napus* also induces cruciferin and napin transcription (Crouch *et al.*, 1985; Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989; Blundy *et al.*, 1991). Other factors such as osmotica (Finkelstein and Crouch 1986; Wilen *et al.*, 1990) and jasmonic acid (Wilen *et al.*, 1991) also induced cruciferin and napin transcript levels in MDEs in comparison to untreated MDEs. In all cases, the proteins accumulate in the zygotic embryos and do not accumulate in the MDEs (Crouch and Sussex, 1981; Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989; Wilen *et al.*, 1990; Blundy *et al.*, 1991; Wilen,

1992). Thus, while MDE systems have been widely used to determine biochemical and molecular changes that may be occurring *in vivo* during zygotic embryogenesis, further studies are required. By using the culture conditions employed by Kennedy (1993) the study of storage protein accumulation in MDEs could be assessed further.

7.0. Objectives.

The processes of germination and early seedling growth are very complex. These processes are further compounded by environmental factors such as low temperature. In *B. napus*, low temperature results in lower germination rates and longer emergence periods (Mills, 1993). Although selection criteria for low temperature growth has been attempted for tomato (Hoek *et al.*, 1993) and *Brassica* (Nam and Park, 1992), the only morphological characteristic tested to date is seed size. Barber *et al.* (1991) determined that seed size is not a selection marker for seed vigor at low temperature within the Westar cultivar.

Although *B. napus* displays genotypic variation there is little room for improvement in nearly isogenic cultivars such as Westar. Indeed selection for enhanced low temperature germination resulted in little success (Barber *et al.*, 1991; Wilson *et al.*, 1992). When dealing with a commercially popular cultivar new means of improving low temperature germination need to be found to increase the length of the growing season and to increase geographic distribution to more northern latitudes.

The environmental effects on low temperature germination have received little attention. No attempt has been made to study underlying biochemical and molecular processes during low temperature germination. The present study is the first known attempt to define some of these processes with the ultimate hope of discerning any biochemical and/or molecular markers that could be used to enhance low temperature germination.

The events during embryogenesis play an important role in the

deposition of reserves (Taylor *et al.*, 1993). Variation in seed meal (Bell and Keith, 1991) and oil content (Shafii *et al.*, 1992) are caused by regional environmental and cultivar differences. These differences would be expected to impact on germination and seedling growth.

Emergence is comprised of sequential developmental processes; germination and early seedling growth. By studying each process independently by traditionally defined morphological, biochemical and molecular markers we hope to gain a better understanding of the underlying processes that contribute to successful germination and subsequent early seedling growth at low temperature. Therefore, our first goal was to determine wether Westar displayed seedlot differences with repect to low temperature germination. Thereafter, physiological, biochemical, and molecular processes were studied during low temperature germination and early seedling growth. In addition, an artificial seed system was employed to study the expression of storage protein genes and accumulation of their end products.

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Chapter 2. Germination and Early Seedling Development Under Low Temperature in *Brassica napus* cv. Westar.

1.0. Introduction.

Seedling emergence is comprised of both germination and early seedling development. Temperature, light, and water are the major environmental factors determining the success of germination and early seedling development (Murray, 1984; Bewley and Black, 1994). Generally, temperatures below the optimum result in progressively poorer germination (Liptay and Schopfer, 1983; Liengsiri and Hellum, 1988; Mohapatra and Suggs, 1989; Steiner and Jacobsen, 1992). Imbibition occurs at low temperature, but damage to embryos or seedlings may prevent germination and growth (Bradbeer, 1988). Suboptimal temperatures may also impair protein synthesis (Meza-Basso *et al.*,1986), limiting the coordination of metabolic processes required for proper germination and early seedling development.

Recommended soil temperatures for planting canola (*B. napus* cv. Westar) are between 15 and 20°C (Anonymous, 1980); however, the mean soil temperature during planting in Alberta ranges from 5 to 10°C. Such temperatures can result in slow and asynchronous seed germination, leading to poor stand establishment and irregular maturation (Stewart *et al.*, 1990; Barber *et al.*, 1991). Further, slow germination increases the time from planting to maturity, predisposing the crop to disease and adverse environmental conditions (Acharya *et al.*, 1983). Seedling growth is also adversely affected by low temperature, further reducing stand establishment (Stewart *et al.*, 1990).

Successful germination at low temperature is not solely a function of temperature. Genotype, growth conditions during seed maturation, storage conditions (Bernal-Lugo and Leopold, 1992; Vertucci, 1992), and seed pretreatment (Nelson *et al.*, 1984; Frisbee *et al.*, 1988; Amritphale *et al.*, 1989; Basra *et al.*, 1989) also influence germination.

Germinating oil seeds rapidly mobilize storage lipids and convert them to sucrose, the primary nutrient during heterotrophic growth (Beevers, 1979; Doman *et al.*, 1982; Trelease, 1984). Prior to this

conversion, enzyme synthesis and organelle assembly is required to produce glyoxylate cycle enzymes, such as MS and ICL, and the glyoxysomes where these enzymes are localized (Breidenbach and Beevers, 1967; Gerhardt and Beevers, 1970). Mobilized storage proteins provide amino acids required for the synthesis of enzymes and structural proteins during germination and early seedling development (Bhandari and Chitralekha, 1984). In *Brassica*, the major storage proteins are napin and cruciferin, which at seed maturity account for 20-30% and 60% of the total protein, respectively (Crouch and Sussex, 1981; DeLisle and Crouch, 1989).

To date, research on low temperature germination in canola has been limited to assessing germination rates under low temperature regimes (Acharya *et al.*, 1983; Kondra *et al.*, 1983; Barber *et al.*, 1991). Acharya *et al.* (1983) determined that 10°C is the best temperature to select for superior low temperature germination in Westar; however, selection for rapid germination at this temperature produced inconsistent results. Similarly, King *et al.* (1986) found that such selections did not necessarily lead to improved performance. These inconsistencies suggest complex control of germination and emergence. In the present study, we characterized physiological changes during seed germination and early seedling development under low temperature in *B. napus* cv. Westar. To this end, seedlots that exhibited extremes in germination potential at 10, 6 and 2°C were selected. ICL activity, lipid mobilization and protein mobilization were studied during both germination and early seedling development in these seedlots.

2.0. Materials and Methods:

2.1. Plant material. Certified seed (Allard, 1960) of canola (*B. napus* cv. Westar), labeled to germinate to 95% at 25°C, was obtained from six different seed growers in Alberta. From germination tests at 10°C, 6°C and 2°C, two seedlots were selected for further study and are denoted as L and H. Seed quality was high in both seedlots; out of one hundred seeds 2.3±0.4 were damaged in L and 2.0±0 were damaged in H.

2.2. Seed germination. Germination was measured at four temperatures: 22°C, 10°C, 6°C, and 2°C. One hundred seeds were placed in 50 mL flasks containing a piece of nylon mesh and 3.5 mL double distilled water. This allowed for 50% coverage of the seed, thereby allowing for unhindered gas exchange while providing adequate water for germination and early seedling growth. The flasks were plugged with cotton to allow for gas exchange with the external air. Germination tests were performed from the onset of imbibition until 95% germination had occurred in the 10°C sample. Germination was defined as occurring once the radicle had emerged 1mm through the seed coat. Percent germination was determined according to Wilson *et al.* (1992).

2.3. Isocitrate lyase assay. Isocitrate lyase (*threo*-Ds-isocitrate glyoxylate lyase; EC4.1.3.1, ICL) was assayed at 22°C according to the methods of Cooper and Beevers (1969). Individual seeds representing specific stages of seedling development (based on measured radicle lengths) and DAI were collected from both seedlots. Single seeds were ground in 300 µL ice cold grinding buffer (Cooper and Beevers, 1969). The slurry was then centrifuged at 4°C for 10 min in a microfuge at 13000 rpm, and 225 µL of the supernatant was drawn off and centrifuged for an additional 5 min at 4°C. The formation of glyoxylate phenylhydrazone (Dixon and Kornberg, 1959) was assayed at 324 nm with a Cary 219 (Varian associates, Palo Alto) spectrophotometer. The molar extinction coefficient was 1.7×10^4 (Cooper and Beevers, 1969).

2.4. Lipid determination. Total lipid was determined gravimetrically

(Johnson-Flanagan *et al.*, 1991). Sixty seeds per sample were dried in a vacuum oven for moisture determination. Samples of 20 seeds were ground in heptane:ethanol (3:1, v/v). The slurry was vortexed and spun for 20 s at 400 x g, the upper organic phase was removed to a preweighed test tube, and the lower organic phase was re-extracted with 2X volume heptane. The upper organic phases were then pooled, dried at 80°C in a vacuum oven and weighed.

2.5. Protein determination. Single seeds were ground in 0.4 M sucrose, 0.165 M Tricine buffer (*N*-tris(hydroxymethyl)methylglycine, Sigma), adjusted to pH 7.5, 0.1 M KCl, 10 mM $MgCl_2$, and 10 mM dithiothreitol. The buffer solubilized proteins (both cruciferin and napin were extracted in the buffer when assessed via SDS-PAGE against Laemmli (1970) buffer) were then quantified according to the method of Bradford (1976) using BSA as a standard.

2.6. Protein analysis. Gradient (7.5-12%) SDS polyacrylamide gels were run for 1 hr at 180 volts in the Bio-Rad Mini-Protean cell electrophoresis apparatus according to the method of Laemmli (1970). Samples of three seeds were ground in 300 µL of warm (65°C) sample buffer (12% glycerol (w/v), 5% 2-mercaptoethanol (v/v) and 2% SDS (w/v) in 62.5 mM Tris-HCl, pH 6.8), heated for 20 min at 65°C, and then centrifuged for 5 min at 13000 X g at room temperature. The supernatant was collected and 10 µL loaded per lane, which was equal to 1/10th the amount of total protein in 1 seeds. The gels were stained with 0.5% Coomassie R-250 in glacial acetic acid:isopropanol:water (1:3:6, v/v/v) and destained in 40% methanol and 10% acetic acid.

2.7. Statistical analysis. Seeds were randomly collected from a population of dry seed and germinated as above. During the experiments, seeds representative of the average developmental stage for each combination of DAI, temperature, amd seedlot were selected for use. In experiments where specific developmental stages were used, seeds were collected randomly from flasks based on radicle length, temperature, and

seedlot. A t-test was used to derive the statistical difference between seedlot sample means with P=0.05 and n=3. Correlation coefficients (r) were generated by plotting a linear least squares fit line through values for the same DAI using Kaleidagraph 2.1 (Synergy Software, Reading). Unless otherwise noted, all values represent the mean of three independent replicates $\pm$ 1 SD.

3.0. Results.

3.1. Seed germination. Both L and H seedlots were certified to exhibit 95% germination at 25°C. Under control conditions of 22°C, both seedlots reached 95% germination by day 4. At 10°C, germination of L increased rapidly, reaching approximately 95% by day 8 (Fig. 2-1), in comparison to H which increased gradually between 2 and 8 d then increased rapidly between day 8 and 10 reaching a maximum of 80% by day 12. Germination rates were low in both seedlots at 6 and 2°C. During germination and seedling growth, visible fungal infection was absent from all samples tested by.

3.2. Seedling growth. H dry seed was larger than L dry seed: 384±4 mg and 310±3 mg, respectively (based on 100 seeds). Plots of fresh weight increases show L growth was similar to H under optimal conditions (22°C) and at 10°C, but L attained a higher mean fresh wt. by day 10 (Fig. 2-2). Fresh weight increases were limited at both 2 and 6°C (data not shown for the 2°C samples). Increases in fresh weight were correlated with increases in germination. Correlation coefficients (r) for L were 1.0 at 22°C, 0.96 at 10°C, 0.99 at 6°C, and 0.68 at 2°C. In comparison, the r values for H were 0.99 at 22°C, 0.97 at 10°C, 0.63 at 6°C, and 0.43 at 2°C.

3.3. Extractable isocitrate lyase activity. Under control conditions of 22°C, both seedlots displayed similar increases in ICL activity, reaching high levels by 2 to 3 DAI. At 10°C, ICL activity in the L seedlot increased gradually over the first 4 DAI, then increased rapidly from day 4 to 8 (Fig. 2-3). There was an approximate three-fold increase in ICL activity in both the 6°C samples relative to the initial ICL activity in the mature seed (0 DAI). By day 6, ICL activity in L at both 10 and 6°C was significantly higher than that measured in the H seedlot at 10 and 6°C (P=0.05) respectively. At 10°C in H, ICL activity increased gradually from day 8 until day 12 (Fig. 2-3). The 6°C sample showed slight increases over the same time frame. When the best fit lines were calculated for both the L and H data, it was found that differences between 22 and 10°C resulted

Figure 2-1. Percent germination at different germination temperatures in *B. napus* cv. Westar. Seed sources tested were L seedlot; (■) 10°C, (●) 6°C, and (▲) 2°C. H seedlot; (□) 10°C, (○) 6°C, and (△) 2°C. Each value represents the mean of three independent replicates ± 1 SD. Analysis proceeded until 95% germination was attained at 10°C for each seedlot.

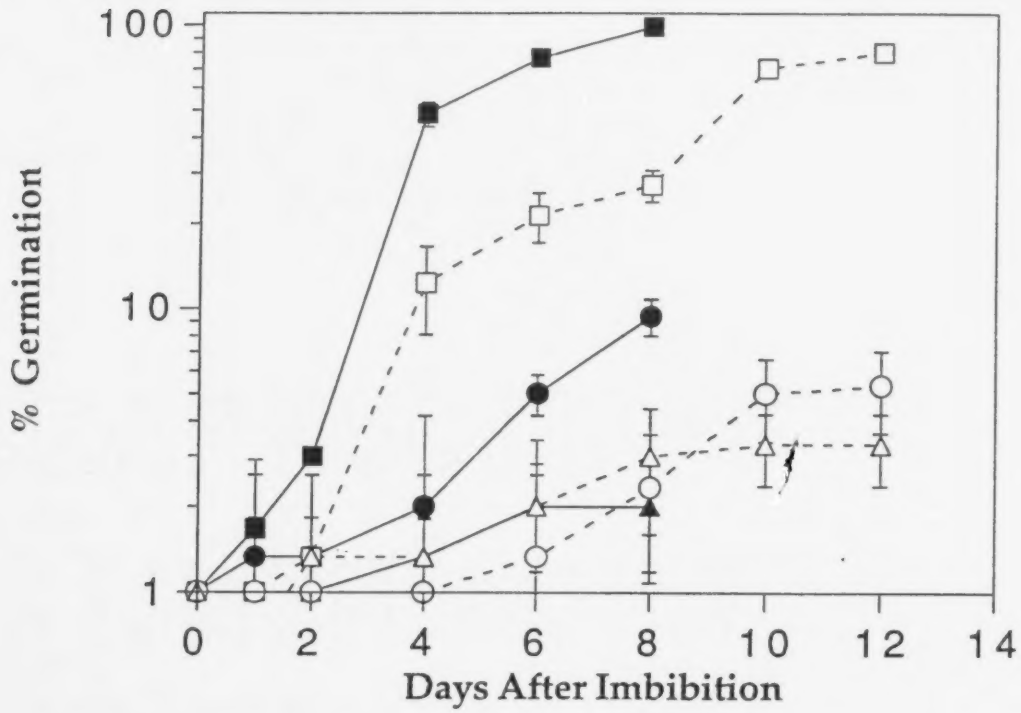


Figure 2-2. Changes in fresh weight during germination and early seedling growth at optimal and suboptimal germination temperatures in two seedlots of *B. napus* cv. Westar. L seedlot; (♦) 22°C, (■) 10°C, and (●) 6°C. H seedlot; (◊) 22°C, (□) 10°C, and (○) 6°C. Each value represents the mean ± 1 SD increase in fresh wt. for 100 seeds of three independent replicates.

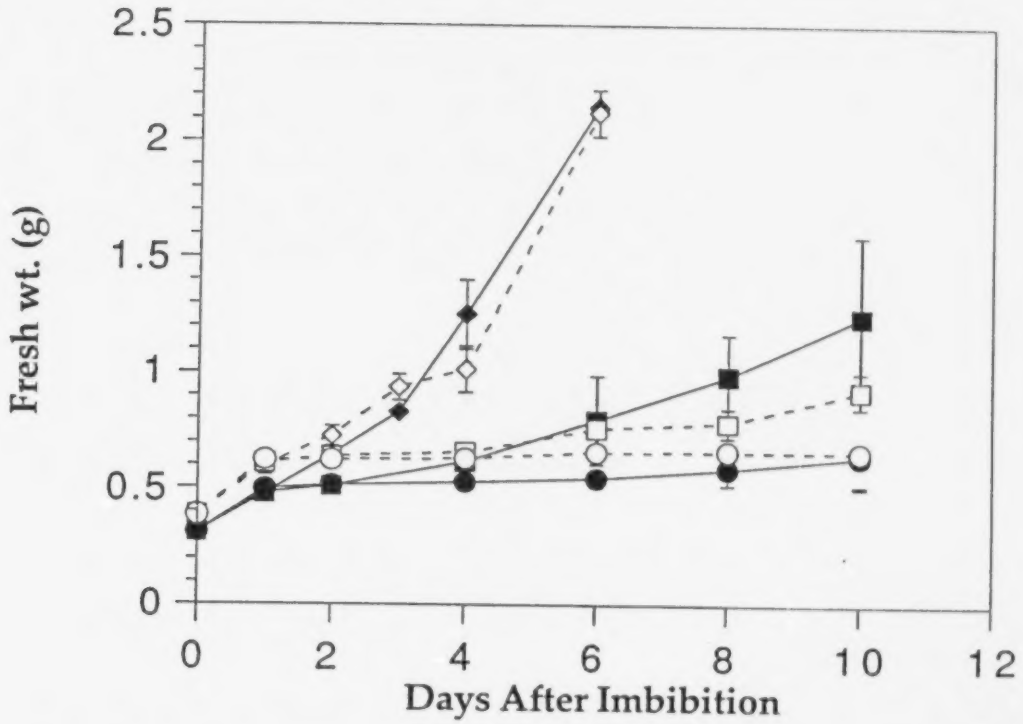
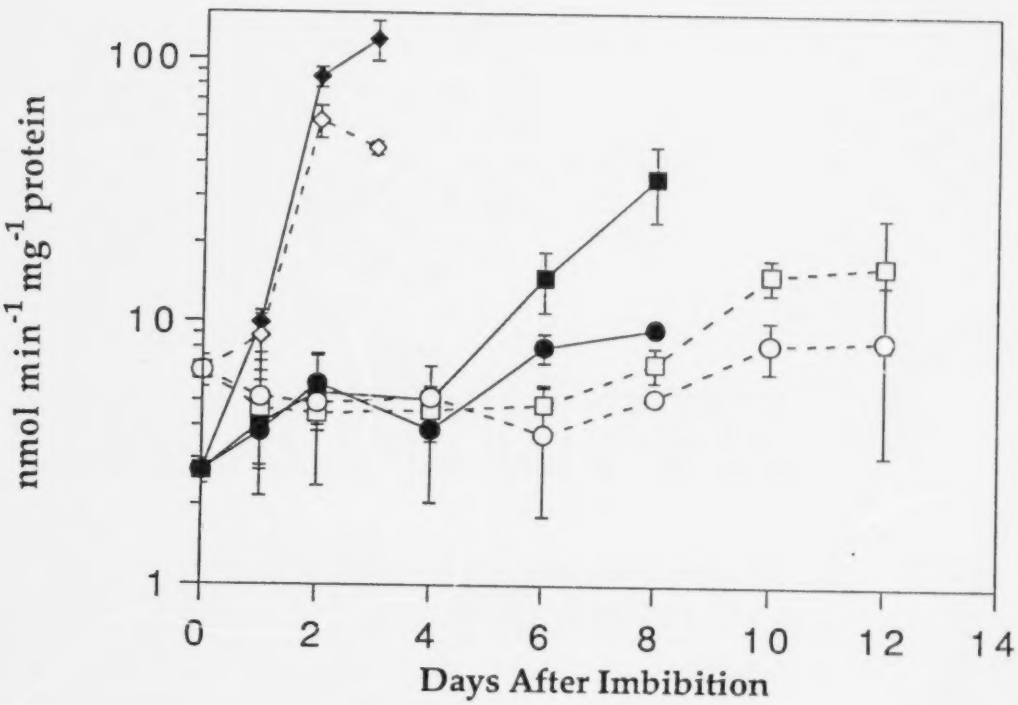


Figure 2-3. Extractable ICL activity during germination and early seedling development in two seedlots of *B. napus* cv. Westar under optimal and suboptimal germination temperatures. ICL activity was assayed at 22°C. L seedlot; (♦) 22°C, (■) 10°C, and (●) 6°C. H seedlot; (◊) 22°C, (□) 10°C, and (○) 6°C. Each value represents the mean of three independent replicates ± 1 SD. Analysis proceeded until 95% germination was attained at 10°C for each seedlot.


100
10
1
0
2
4
6
8
10
12
14
nmol min-1 mg-1 protein
Days After Imbibition

from a temporal shift in activity while differences between 22 and 6°C resulted from both a temporal shift and a reduction in the slope of the line for H (slopes for L at 22, 10 and 6°C were 0.95, 0.88, and 0.90 respectively, and for H at 22, 10, and 6°C were 0.83, 0.82, and 0.57 respectively).

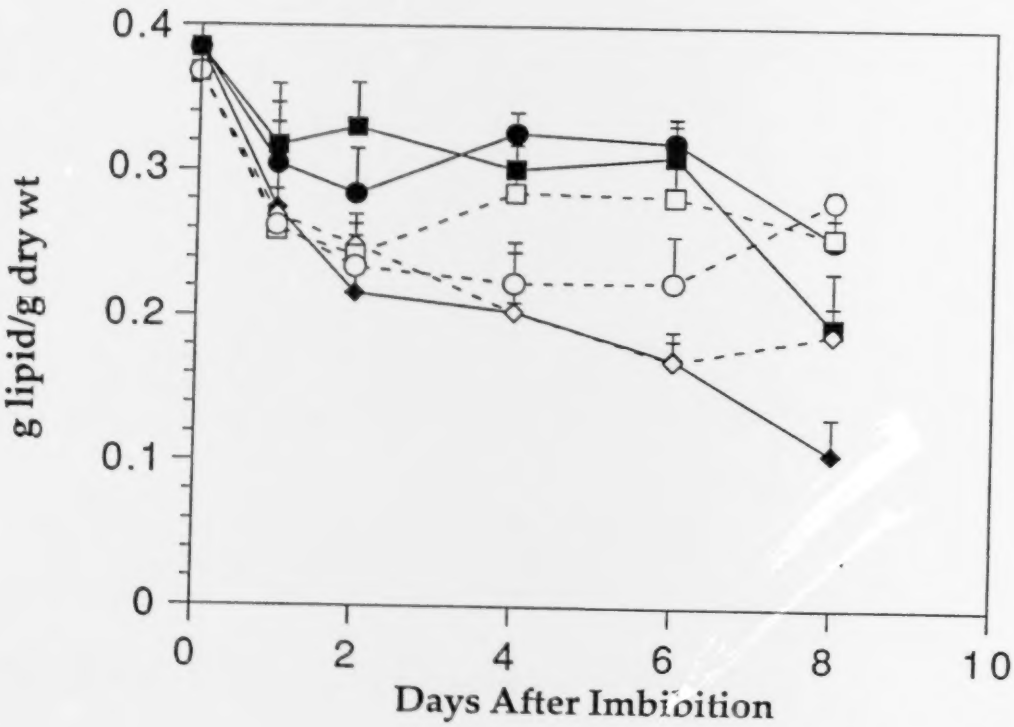
ICL activities were also assayed at different stages of development during early seedling growth (Table 2-1). The 6°C samples display higher levels of ICL activity relative to the 10°C samples. In addition, 22°C samples displayed much higher levels of ICL activity in both seedlots. There were, however, no significant differences in ICL activity between the two seedlots during seedling growth at all temperatures (P=0.05). Slopes for ICL activity during stages of seedling growth revealed that between 22 and 10°C differences resulted from a temporal shift in activity, while differences between 22 and 6°C resulted from both a temporal shift and a reduction in the slope of the lines of both L and H (slopes for L at 22, 10, and 6°C were 0.93, 0.94, and 0.65 respectively, and for H at 22, 10 and 6°C, were 0.99, 0.82, and 0.74 respectively). Since the ICL activity in H peaks at 0.2 cm radicle length, and thereafter slowly declines in activity the calculated slope was derived from the initial increase from mature seed to 0.2 cm radicle.

3.4. Lipid mobilization. Mature seed from both seedlots contained approximately the same amount of total lipid (Fig. 2-4). In all cases, lipid content dropped within the first day of imbibition. At 22°C, lipid mobilization was similar in L and H until day 6, after which H leveled off and L continued to decrease in total lipid. In the L seedlot both the 10 and 6°C samples decreased in total lipid after 6 DAI. These differences were not noted at 10 and 6°C in H. Decreases in total lipid were inversely proportional to increases in ICL activity. Correlation coefficients (r) for L were -0.86 at 22°C, -0.90 at 10°C, and -0.73 at 6°C. In comparison, the r values for H were -0.72 at 22°C, -0.42 at 10°C, and -0.85 at 6°C.

Table 2-1: Isocitrate lyase specific activity in seedlings from two seedlots, L and H, at optimal and suboptimal temperatures. Values represent the means of three separate samples ± 1 SD. * denotes a significant difference between seedlots with P=0.05. ‡ these values represent the mean of two independent samples.

Temperature	Radicle Length	Seedlot	
		L	H
	-cm-	nmol min^{-1} mg^{-1} protein	
22°C	0.1	12 ± 1	48 ± 6*
	0.2	53 ± 10	78 ± 4
	0.5	68 ± 5	68 ± 2
	1.0	95 ± 2	61 ± 7*
10°C	0.1	6 ± 1	4 ± 1
	0.2	7 ± 1	8 ± 2
	0.3	8 ± 2	8 ± 1
	0.5	8 ± 2	8 ± 1
	1.0-1.5	17 ± 3	11 ± 2
6°C	0.1	13 ± 2	12 ± 2
	0.2	12 ± 4	12 ± 2
	0.3	15 ± 2	9 ± 3
	0.5	15 ± 3	9‡
	1.0-1.5	15 ± 1	17‡

Figure 2-4. Lipid degradation during germination and early seedling growth under optimal and suboptimal germination temperatures in two seedlots of *B. napus* cv. Westar. Total lipid was determined gravimetrically. L; (♦) 22°C, (■) 10°C, and (●) 6°C. H; (◇) 22°C, (□) 10°C, and (○) 6°C. Each value represents the mean + 1 SD of three independent replicates.



3.5. Total soluble protein. Total buffer-soluble protein for 100 seeds from each seedlot are compared in Table 2-2. It is apparent that H and L possess similar amounts of protein in the mature seed. At 22°C there was an initial rapid breakdown of protein in both seedlots, followed by a steady decrease until day 6. At 10°C and 6°C, buffer-soluble protein steadily decreased. Similar gradual decreases in protein were noted at 2°C in both seedlots (data not shown).

3.6. Storage protein mobilization. Cruciferin, the major storage protein in canola, is composed of six subunits with molecular weights between 29 to 37 kD and 22 to 24 kD. Changes in cruciferin (5 subunits can be reliably discerned) were first noted in L after day 3 at 22°C, day 6 at 10°C and day 8 at 6°C (Fig. 2-5A, 2-6A, and 2-7A). In comparison, there was little cruciferin mobilization by day 4 and day 8 for H at 22°C and 10°C, respectively (Fig. 2-5A and 2-6A). No cruciferin breakdown was discernable in the 6°C H sample (Fig. 2-7A), nor were changes noted in either seedlot over a 10 day period at 2°C (Fig. 2-8).

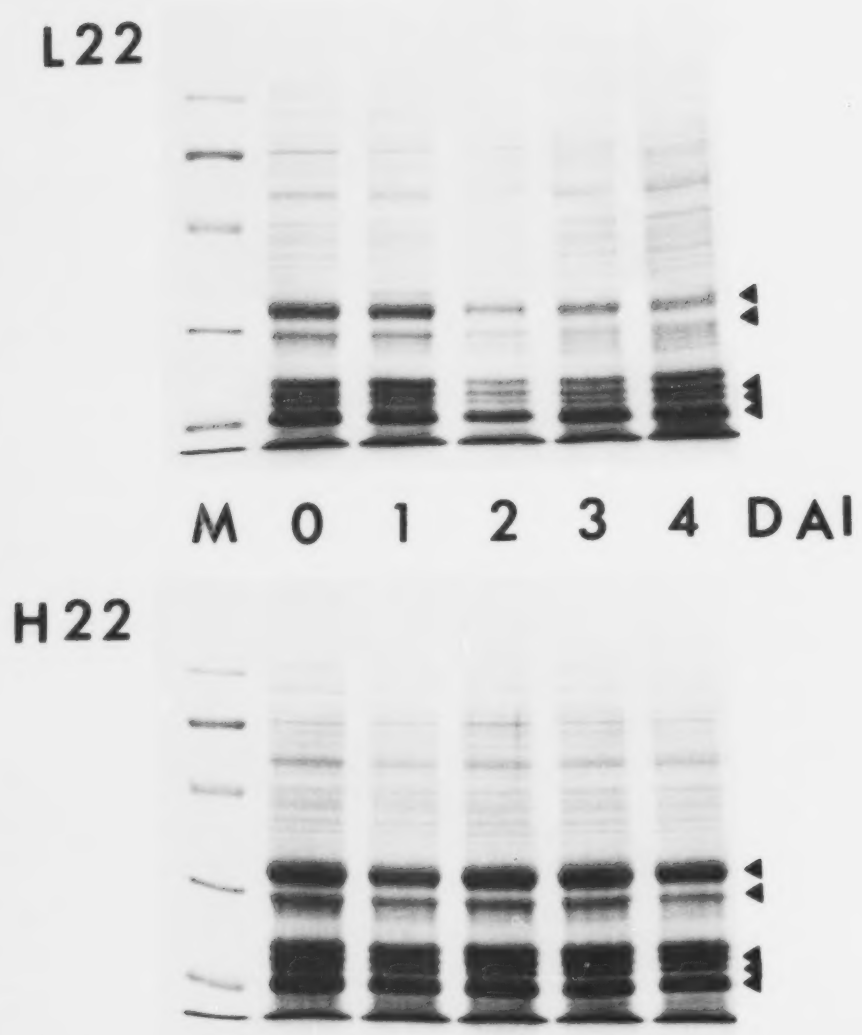
Protein profiles were also compared at different stages of seedling development, with L mobilizing cruciferin protein at 5 mm in the 22°C and the 10°C samples, and at 3 mm in the 6°C sample (Fig. 2-5B, 2-6B and 2-7B). In comparison, there was little or no mobilization in any of the H samples (Fig. 2-5B, 2-6B, and 2-7B). In each of these profiles, L was observed to decrease in cruciferin to a greater extent in comparison to H. The 2°C condition was not tested for cruciferin mobilization at different stages of postgerminative growth.

Table 2-2. Total buffer soluble protein content for 100 seeds of two seedlots, L and H, of *B. napus* cv. Westar during germination and early seedling growth at optimal and suboptimal temperatures. Each value is the average of three independent determinations ± 1 SD. * denotes significant difference between seedlots with P=0.05.

Temperature	DAI	Seedlot	
		L	H
	cm	mg	
Control (22°C)	0	52 ± 9	62 ± 2
	1	23 ± 4	42 ± 3*
	2	20 ± 2	28 ± 4
	3	11 ± 1	23 ± 5
	4	10 ± 3	22 ± 6
	6	17 ± 3	11 ± 8
10°C	0	52 ± 9	62 ± 2
	1	47 ± 13	69 ± 4
	2	30 ± 6	53 ± 8
	4	20 ± 4	38 ± 6*
	6	17 ± 7	38 ± 4*
	8	26 ± 6	22 ± 5
6°C	0	52 ± 9	62 ± 2
	1	37 ± 14	66 ± 7
	2	38 ± 2	46 ± 8
	4	46 ± 2	29 ± 4*
	6	22 ± 3	27 ± 5
	8	24 ± 7	34 ± 3

Figure 2-5. Protein profiles of *B. napus* cv. Westar seed from two seedlots, L and H, germinated at 22°C. SDS-soluble protein was extracted and gels were loaded on a per seed basis. Molecular weight markers (M) were from top to bottom: 97.4 kD, 66.2 kD, 42.3 kD, 31.1 kD, 21.5 kD, and 14.4 kD. (A) At 0 to 4 days after imbibition (DAI). (B) At 0 to 5 mm radicle protrusion. (◂) denotes cruciferin subunits.

A.



B.

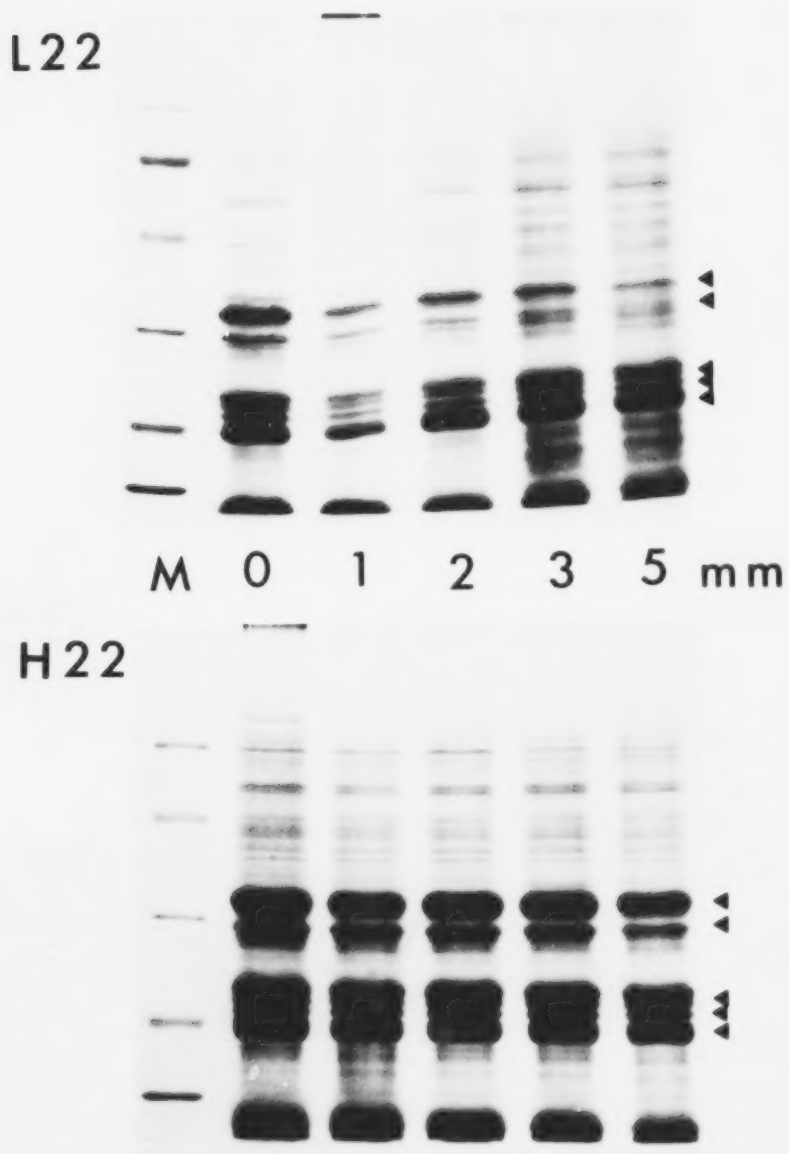
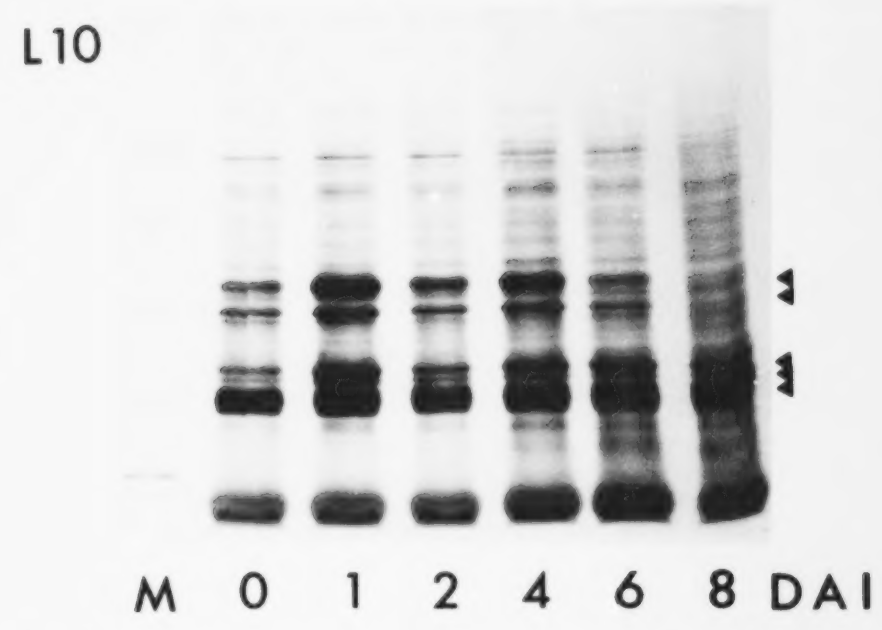


Figure 2-6. Protein profiles of *B. napus* cv. Westar seed from two seedlots, L and H, germinated at 10°C. SDS-soluble protein was extracted and gels were loaded on a per seed basis. Molecular weight markers (M) were from top to bottom: 97.4 kD, 66.2 kD, 42.3 kD, 31.1 kD, 21.5 kD, and 14.4 kD. (A) At 0 to 8 days after imbibition (DAI). (B) At 0 to 5 mm radicle protrusion. (◂) denotes cruciferin subunits.

A.



B.

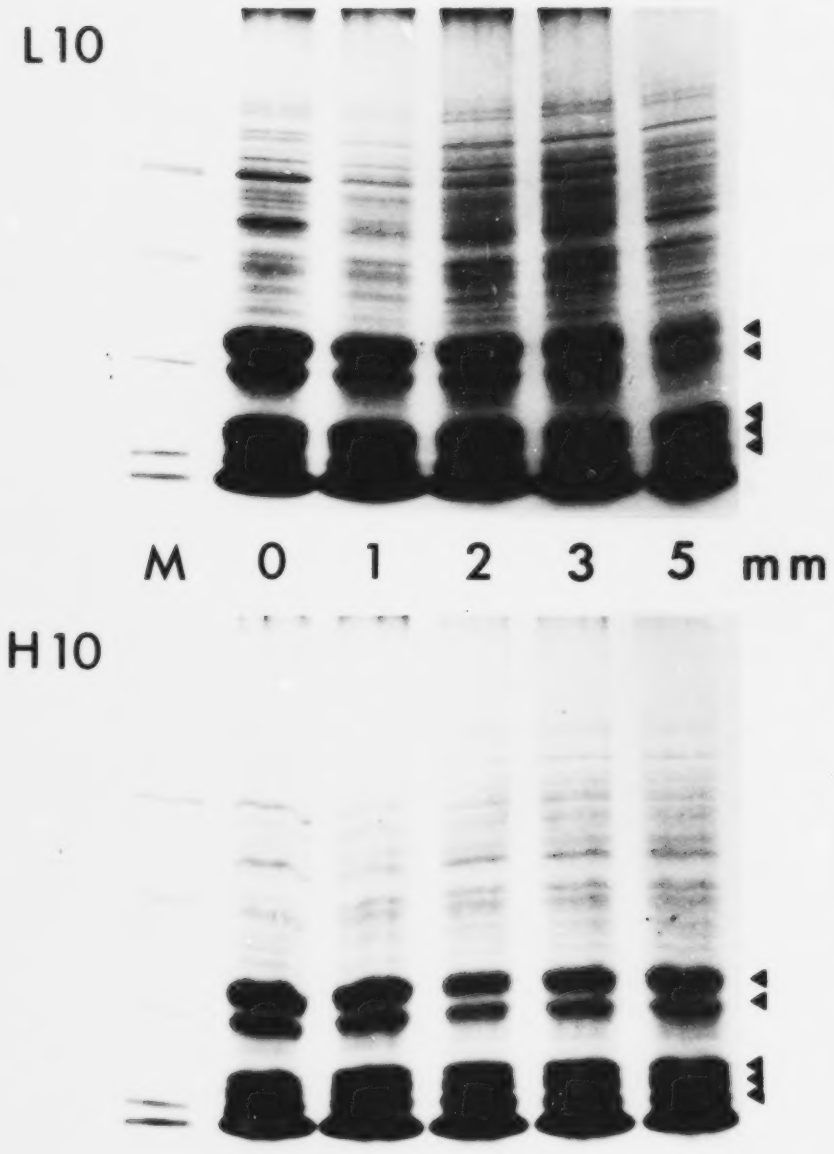
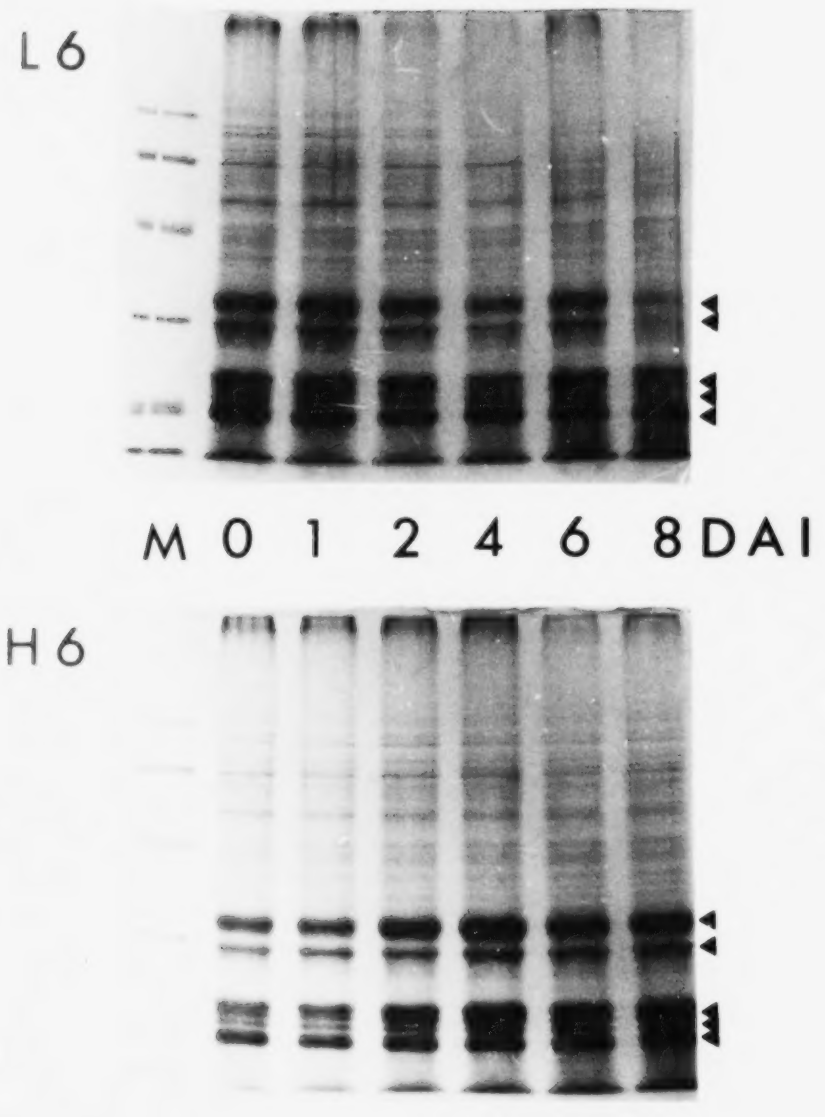


Figure 2-7. Protein profiles of *B. napus* cv. Westar seed from two seedlots, L and H, germinated at 6°C. SDS-soluble protein was extracted and gels were loaded on a per seed basis. Molecular weight markers (M) were from top to bottom: 97.4 kD, 66.2 kD, 42.3 kD, 31.1 kD, 21.5 kD, and 14.4 kD. (A) At 0 to 8 days after imbibition (DAI). (B) At 0 to 5 mm radicle protrusion. (◂) denotes cruciferin subunits.

A.



B.

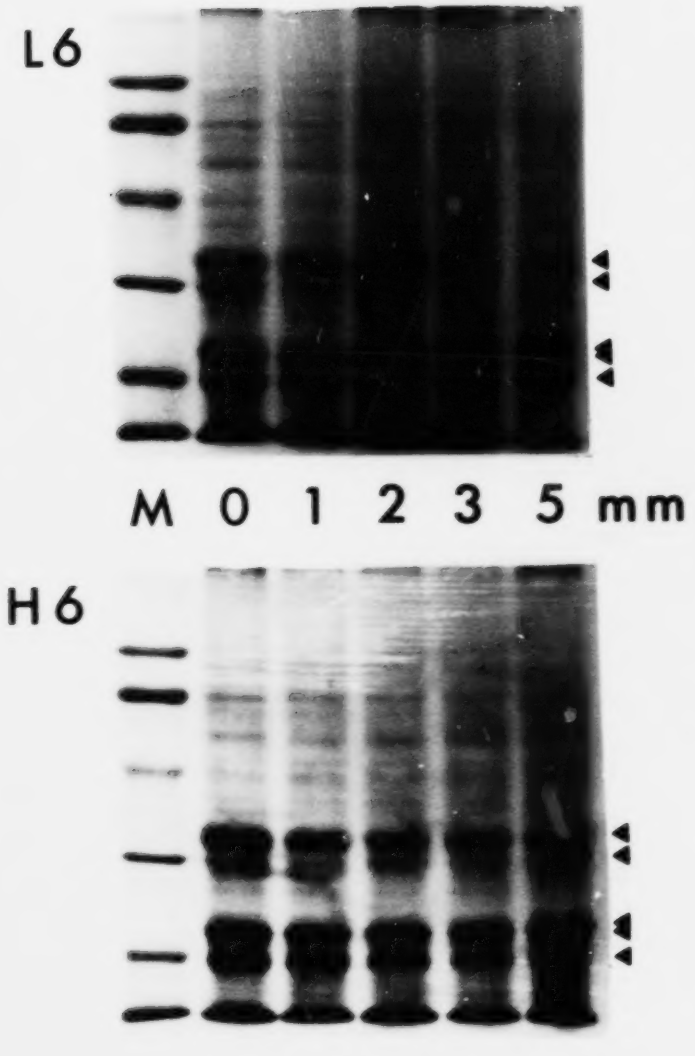
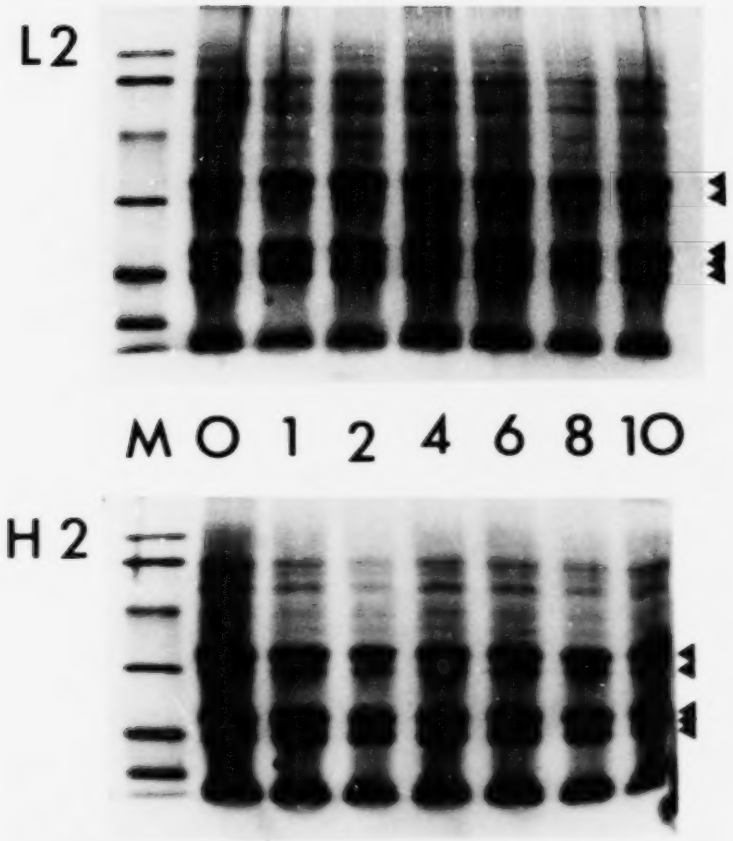


Figure 2-8. Protein profiles of *B. napus* cv. Westar seed from two seedlots, L and H, germinated at 2°C over the course of imbibition (DAI). SDS-soluble protein was extracted and gels were loaded on a per seed basis. Molecular weight markers (M) were from top to bottom: 97.4 kD, 66.2 kD, 42.3 kD, 31.1 kD, 21.5 kD, and 14.4 kD.


L2
M O 1 2 4 6 8 10
H 2

4.0. Discussion.

Studies show that canola can germinate in temperatures ranging from 2 to 25°C (Canola Growers Manual, 1991); however, temperatures below 10°C result in low germination (Acharya *et al.*, 1983; Barber *et al.*, 1991), and longer germination times (Kondra *et al.*, 1983; King *et al.*, 1986). In the present study, both seedlots germinated to completion (95%) within 4 days under control conditions (22°C). At 10°C, both seedlots reached high levels of germination, but germination rates were slower. At temperatures below 10°C, germination was low. A similar relationship between seedling growth and temperature was noted. For Westar, the baseline growth temperature has been determined to be about 5°C (Morrison *et al.*, 1989). From the seedlots tested it is apparent that differences in germination and seedling growth exist within the cultivar Westar. L displayed a higher and faster germination rate at 10°C. These results were mirrored by the increases in fresh weight with L growing at a faster rate in relation to H during early seedling growth. These observations are reflected in high correlation coefficients between germination potential and fresh weight in the L over the temperature range studied, but only at 22°C and 10°C in H, suggesting that L has better seedling growth at the suboptimal temperatures in comparison to the H seedlot.

Germinating oil seeds rely on reserves (around 60% of total seed weight) of lipids until growth can be maintained through photosynthesis. The glyoxylate cycle enables germinating oil seeds to bring about a net conversion of oils (triglycerides) to carbohydrates (Trelease, 1984; Mathews and Van Holde, 1990). ICL is a glyoxylate cycle enzyme that converts isocitrate to glyoxylate and succinate (Vanni *et al.*, 1990). Therefore, by monitoring the ICL activity we were able to estimate the overall extractable cell free activity of the glyoxylate cycle during and after germination.

ICL activity in sunflower and cotton is present in the mature seed and increases activity within 2 to 3 DAI (Allen *et al.*, 1988); whereas, in

castor bean the ungerminated seed has low levels of ICL (Gerhardt and Beevers, 1970). In *Brassica*, ICL activity is low in the mature seed (Johnson-Flanagan *et al.*, 1992), and upon imbibition reaches high levels of activity at 3 DAI (Comai *et al.*, 1989; Dietrich *et al.*, 1989) then decreases by 4 to 6 DAI (Ettinger and Harada, 1990) in seedlings. In the present study both L and H reached high levels of ICL activity at 22°C between 2 to 3 DAI, and, therefore, followed the same temporal pattern of accumulation (Fig. 2-3). Under suboptimal conditions (10 and 6 °C), however, ICL activity (assayed at 22°C) was much lower, but appears to follow a pattern that mirrored increases in germination rates. Because the assay for ICL was performed *in vitro* at 22°C it is important to consider that *in vivo* the enzyme activity could be lower at the suboptimal temperature. Polanowski and Obendorf (1991) demonstrated that purified ICL from soybean, which shares a high amino acid homology to *B. napus*, maintained activity *in vitro* between the temperatures of 50°C To -12 °C. Therefore, in the present study it is concievable that the ICL activity displayed at the suboptimal temperatures *in vitro* reflects the ICL activity *in vivo*. Lower temperatures cause a temporal shift in the ICL activity at 10°C in both seedlots, but at 6°C the activity is reduced as well as temporally delayed in the H seed.

ICL activity was also monitored during seedling growth. Germination can be defined as radicle protrusion through the seedcoat (Kondra *et al.*, 1983). Radicle emergence ends germination (Bewley and Black, 1994), and therefore, may be considered the first visible sign of seedling growth and development. Once again, high levels of ICL activity were reached at 22°C and these remained significantly (P=0.05) higher than levels under the suboptimal temperatures (Table 2-1). L displayed similar levels of ICL activity during seedling growth at the suboptimal conditions. This suggests that once germination has occurred, ICL activity in the two seedlots is comparable when based on seedling development.

There is a known relationship between ICL activity and lipid

mobilization. At 30°C, the utilization of lipid reserves in cotton seeds begins at approximately 16 hours after imbibition, concurrent with an increase in ICL activity (Doman *et al.*, 1982). In *B. oleracea* seed germinated at 25°C, lipid mobilization occurs 2 DAI, decreasing rapidly then leveling off (Qouta *et al.*, 1991). Cotyledons from *B. napus*, L. var. Dwarf Essex germinated at 25°C were found to rapidly metabolize lipid in 2 to 4 day old seedlings, then decreased steadily through to day 10 (Lin and Huang, 1983). In our study, total lipid was mobilized rapidly after imbibition at 22°C and decreased steadily in both the L and H seedlots (Fig. 2-4). These decreases in total lipid were concurrent with the rapid increase in ICL activity at 22°C in both seedlots (Fig. 2-3) r=-0.85 for L and r=-0.72 for H, and therefore, were consistent with the findings of Lin and Huang (1983). At suboptimal temperatures, lipid mobilization in the L seedlot was delayed until 6 DAI; at which time there was a large decrease in lipid (Fig. 2-4). In the H seedlot this decrease in the total lipids did not occur even up to 8 DAI at 10 and 6°C. Therefore, L displayed an increase in ICL activity at 4 DAI followed by a decrease in lipid reserves 6 DAI at 10°C (r=-0.90). The 6°C sample followed this same trend, but the correlation (r=-0.73) was not as good as in the 10°C sample. Comparatively, ICL activity in H increased gradually, and this increase was not followed by large decreases in lipid reserves at 10°C (r=-0.42). At 6°C ICL activity gradually increased and lipid reserves slightly decreased thereby maintaining a high correlation (r=-0.84). Thus, there was an inverse relationship between ICL and lipid mobilization except at 10°C in H, where there was less lipid mobilized than was predicted from the ICL activity. This may suggest that lipid mobilization is impaired in H, or that ICL is inhibited *in vivo*, and may contribute to the observed decrease in germination potential at low temperature. These results may also suggest that metabolite transfer between organelles (glyoxysomes and mitochodria) and their transfer through the cytoplasm may be restricted at low temperature, and differences may exist between the seedlots in respect

to metabolites and\or necessary enzymes are limited.

Further, Qouta *et al.* (1991) found that decreases in lipids were preceded by a decrease in total protein. Decreases in buffer-soluble proteins occured prior to lipid degradation in the 10°C and 6°C samples for both seedlots. In *B. oleracea*, total protein began to decrease 1 day after imbibition and continued to drop throughout early seedling growth (Qouta *et al.*, 1991). In the present study, there was a direct relationship between buffer-soluble protein mobilization and temperature, but only L displayed decreases in buffer-soluble proteins prior to lipid degradation (Table 2-2).

In order to study the mobilization of specific storage proteins we examined protein changes by SDS-PAGE. The major storage protein in *Brassica* is cruciferin, a 12S globulin holoprotein composed of 6 subunits, with a molecular weight of 300 kD (Rodin and Rask, 1990). There are 4 α polypeptides which range in molecular weight from 29 kD to 37 kD, and 4 β polypeptides which range from 22 kD to 24 kD (Dalgalarrondo *et al.*, 1986; Rodin and Rask, 1990). The other major storage protein in *Brassica* is napin (DeLisle and Crouch, 1989).

Cruciferin accounts for approximately 60% of the total protein in the mature seed, and therefore its degradation can be followed via SDS-PAGE analysis (Crouch and Sussex, 1981). Cruciferin mobilization occurs after germination and proceeds rapidly under optimal conditions (Murphy *et al.*, 1989; Hoglund *et al.*, 1992). In the present study mobilization occurred rapidly under control (22°C) conditions (Fig. 2-5A), but was delayed temporally under the suboptimal conditions (Fig. 2-6A, 2-7A) in both seedlots. There was, however, no variation in the pattern of breakdown, i.e., the higher molecular weight subunits were preferentially degraded. This observation is consistent with proposed orientation of the hydrophilic c-terminus of the alpha-subunit situated at the surface of the cruciferin molecule (Pleitz *et al.*, 1987; Robin *et al.*, 1991), and therefore would undergo mobilization prior to the internal hydrophobic

regions of the beta-subunits. Cruciferin breakdown in the L seedlot was correlated with the other metabolic processes occurring during early seedling growth such as lipid mobilization and increases in ICL activity. Again, the H seedlot did not display the same correlations. These differences may suggest that amino acids necessary for proper early seedling development are not available in the H seedlot, and therefore, the germinating seed is not supplied with essential proteins required during this essential stage of development.

In the present study we have shown that variations in germination rates and early seedling growth exist under low temperatures in Westar. It is apparent that L, in comparison to H, germinates more rapidly and has exceptional early seedling growth. Based the parameters tested, it appears that perhaps proper coordination between storage reserve mobilization, storage protein utilization and development are paramount in determining the success of superior germination at low temperature within the cultivar Westar. In addition, even if these processes are fully coordinated the entire process may be simply delayed. Alternatively there may be other rate limiting reactions or processes involved which were not revealed in the present study. Finally, the fact that seedlots are certified to have 95% germination at 25°C does not indicate that they will all perform the same at suboptimal temperatures.

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Chapter 3. Storage Reserve Mobilization During Low Temperature Germination and Early Seedling Growth in *Brassica napus* cv. Westar.

1.0. Introduction.

To date, research on low temperature emergence in canola has been restricted to assessing germination rates and seedling growth (Acharya *et al.*, 1983; Kondra *et al.*, 1983; Barber *et al.*, 1991; Wilson *et al.*, 1992; Mills, 1993) under low temperature regimes. Generally, these studies reveal that germination rates are reduced at low temperature. The present study was designed to examine the effect of low temperature on specific stages of germination and early seedling growth, to determine the cause of delays (whether thermal or developmental), to relate delays to seedling physiology, and to then recapitulate seedling emergence to give a clear picture of the effect of low temperature at specific developmental stages.

Germination is the developmental period that begins with water uptake and ends with radicle emergence through the seed coat (Bewley and Black, 1994). Prior to imbibition, canola seeds have a very low hydration level and low metabolic activity (Kuras, 1984). Canola seed requires 60-75% moisture content between the temperatures of 2-25°C, in the presence of oxygen, to germinate (Canola Growers Manual, 1991).

The process of germination is subdivided into three phases (Simon, 1984; Vertucci, 1989; Bewley and Black, 1994). During phase I, rapid hydration (imbibition) of the seed occurs and metabolism begins. A lag follows during phase II, while major metabolic events take place in preparation for radicle emergence. The final event, phase III, is characterized by radicle elongation, and marks the end of germination.

Low temperature slows the rate of imbibition (Vertucci, 1989), and may damage embryos, preventing germination (Bramlage *et al.*, 1979; Bradbeer, 1988). This in turn may result in slow, asynchronous germination, leading to poor stand establishment (Stewart *et al.*, 1990; Barber *et al.*, 1991), and ultimately, reduced yield.

Germination ends as the radicle elongates slowly by cell expansion, followed by rapid growth and cell division (Esau, 1977; Kuras, 1986; Kuras, 1987; Bewley and Black, 1994). After germination, water uptake

increases and storage reserves are utilized to support seedling growth. Once the efficient breakdown of the storage reserves is underway the energy is utilized to produce the photosynthetic machinery necessary to support and maintain autotrophic growth. This includes the synthesis of chlorophyll (Callis, 1995).

In canola, there are two types of storage protein, albumins and globulins. These provide the necessary amino acids required for the *de novo* synthesis of proteins after germination such as those require for cellular housekeeping and those involved in the further breakdown of storage reserves. The 1.7S albumins are called napin, and the 12S globulins are called cruciferin (Lonnderal, 1972; Crouch and Sussex, 1981; Rodin and Rask, 1990). The major storage protein, cruciferin, accounts for approximately 50-60% of the total seed protein (Crouch and Sussex, 1981; Hoglund *et al.*, 1992), and is composed of six subunits (Plietz *et al.*, 1987). Each subunit is composed of a heavy α-polypeptide (ranging from 29-37 kD) disulfide bound to a lighter β-polypeptide (ranging from 22-24 kD) (Dalgalarrondo *et al.*, 1986; DeLisle and Crouch, 1989; Rodin and Rask, 1990).

In canola, lipids are mobilized and converted to sucrose, which serves as the primary nutrient source during heterotrophic growth (Beevers, 1979; Doman *et al.*, 1982; Trelease, 1984). As lipids are degraded they are shunted through the glyoxylate cycle eventually giving rise to glyoxylate and succinate (Trelease, 1984; Mathews and Van Holde, 1990). Succinate is converted to sucrose via the TCA cycle and gluconeogenesis (Mathews and Van Holde, 1990). The sucrose can then be utilized by the germinated seed to provide ATP through both substrate level and oxidative phosphorylation (Mathews and Van Holde, 1990). Via these processes decarboxylations in the TCA cycle result in the evolution of CO_2.

The enzyme ICL in the glyoxylate cycle converts isocitrate to glyoxylate and succinate (Vanni *et al.*, 1990) as free fatty acids from

the lipid reserves are mobilized. Concomitant with the depletion of the oil reserves from oil bodies is the degradation of the oil body proteins (oleosins) (Huang, 1992).

Previously we suggested that improper coordination of storage reserve mobilization could account for the observed delays and reductions in germination and early seedling growth (see chapter 2). In the present study germination and early seedling growth are assessed in *B. napus* cv. Westar. Thereafter, specific developmental stages of early seedling growth were studied in relation to storage reserve mobilization.

2.0. Materials and Methods:

2.1. Plant material. Certified seed (Allard, 1960) of canola (*B. napus* L. var. *oleifera* cv. Westar), previously designated Lendholm (chapter 2), was used for the study. Lendholm displayed superior low temperature emergence in comparison to Holmstrom at low temperature, and therefore developmental processes involoved in storage reserve mobilization may reveal limiting processes that could be altered to increase low temperature emergence. The seed was of high quality with only 2.3±0.4% broken seed coats and exposed embryos. The mature seed had a low moisture content (1.63±0.34 %), and was bagged and stored at 4°C in a bell jar.

2.2. Seed germination and seedling growth. Germination tests were carried out according to the protocol outlined in chapter 2. This involved imbibing populations of 100 seed in 50 mL flasks containing a piece of nylon mesh and 3.5 mL of double distilled water. This allowed for unhindered gas exchange while providing adequate water for germination and early seedling growth to occur. The flasks were plugged with cotton to allow gas exchange with the external air. Seeds were maintained at constant temperatures of 22, 10, 6, and 2°C in constant temperature water baths in a 12 hr light/dark photoperiod. Germination was scored as radicle protrusion through the seedcoat, and germination percentage was determined according to Wilson *et al*. (1992). Fresh weight increases were also recorded.

Previously we observed no germination or seedling growth at 2°C (Figure 2-1; chapter 2). The lowest temperature limit for germination in *B. napus* is 2°C (Canola Growers Manual, 1991; Bedi and Basra, 1993), and therefore, 2°C served as the baseline temperature for germination and seedling growth. This enabled us to standardize thermal time for each condition by the following equation: DD=(X-2°C)(DAI), where DD=Degree days, X=temperature conditions (22, 10, or 6°C), and DAI=Days After Imbibition. Radicle lengths of individual seed (from sample sets of 100 seed) were also measured to provide an index of seedling growth for each day after

imbibition (DAI). These values were then converted to DD and tabulated (Table 3-1). From the obtained values we interpolated and derived corresponding values for equivalent DD at each of the temperatures. This allowed another estimation of seedling growth at the different temperatures in relation to thermal degree days. By standardizing thermal time differences between the temperature conditions would reflect differences in processes dependent on temperature. Thereafter, individual seedlings at specific radicle lengths were studied to verify the validity of the preliminary comparisons, thus allowing us to examine specific developmental stages after germination. Seedlings possessing a 15 mm radicle length were chosen as the upper limit because thereafter seedlings exhibit epicotyl growth and greening. During the germination tests, all samples were free of visible fungal infection. Sampling of the 10 and 6°C seed ceased at 64 and 40 DD, respectively, as seedling growth was slow.

2.3. CO_2 determinations. Samples were collected from the flasks with a 1cc syringe. After drawing off 1.1cc from the flask, the loss of air or exchange of gas with the ambient air was prevented by placing a plunger on the end of the needle. Samples were then analyzed on a 5880 Series Gas Chromatograph (Hewlett-Packard) against a 350 ppm CO_2 standard. The total area of the peaks with a retention time of 1.440 were collected and averaged.

2.4. Chlorophyll determinations. Seed (100 from each DAI) was collected from each temperature condition and chlorophyll extracted and determined according to the methods of Moran and Porath (1980), using purified Chl a and Chl b (Sigma) as a standard. Samples were ground in liquid N_2, transferred to 15 mL Corex tubes containing 5 mL of N,N-Dimethylformamide and stored for 24 hrs in the dark at 4°C. Prior to spectrophotometry, samples were centrifuged at 10 000xg for 10 min at 4°C in a Sorval centrifuge with a SS34 Sorval rotor and the supernatant collected. Chl a and b were then determined specrophotometrically at 649 and 665 nm, respectively, on a Cary 219 spectrophotometer (Varian Associates, Palo

Alto, CA.). All operations were carried out under a green safelight to avoid chlorophyll photo-degradation.

2.5. *In vivo* labelling. Samples were randomly selected from seed at each temperature at 1,4,8, and 10 DAI. The samples were imbibed in double distilled water containing $7.4x10^3$ Bq ^{35}S-labelled methionine (Amersham). Mature seed was labelled at 22°C. Labelling was allowed to proceed for 40 degree hours to standardize thermal time between the temperature conditions (ie. 2hrs, 5hrs, and 10hrs for seed imbibed at 22, 10, and 6°C, respectively). ^{35}S-methionine incorporation was terminated by rinsing samples with double distilled water, and placing them in liquid N_2. Samples were then ground in Laemmli (1970) buffer (see SDS-PAGE section) on ice, microfuged for 5 min at 13 000 rpm, and the supernatant collected and placed in a sterile tube. Incorporation was quantified by spotting 10µL aliquots on Whatman glass micro fibre filters (GF\C) air drying for 30 min, and washing in cold 10% TCA, followed by boiling in 5% TCA and 0.2% casein hydrolysate for 15 min. Thereafter, the disks were rinsed three times in cold 95% ethanol. After samples had air dried, incorporation was measured by fluorescence on an Isocap\300 (Des Plaines, Illinois) liquid scintillation counter by placing spotted samples in 5 mL Econofluor (DuPont, Boston, Massachusetts). Protein concentrations were obtained as described in SDS-PAGE section. Values were converted to degree days for the different temperatures. Thereafter, interpolated values were derived enabling comparisons between the different temperatures on the basis of equivalent DD. Sampling of the 10 and 6°C samples ceased after 80 and 40 DD, respectively, as seedling growth was slow.

2.6. SDS-PAGE. Mature seed and seedlings were collected from each temperature during specific stages of development (20-30 seedlings for each stage). Samples were ground in 300 µL hot (65°C) Laemmli (1970) buffer (120mL L^{-1} glycerol, 50 $mL^{-1}L$ 2-mercaptoethanol, 0.05% (w/v) bromophenol blue, and 20g L^{-1} SDS in 62.5 mM Tris-HCl, pH 6.8) in a mortar

and pestle on ice. Samples were then microfuged (13 000 rpm) for 5 min at 4°C, the supernatant retrieved, and placed in a sterile microfuge tube on ice.

Total protein concentration was determined by precipitating a 25 µL aliquot of the supernatant with 50 µL of cold TCA (10%) in a sterile microfuge tube. The samples were microfuged (13 000 rpm) for 10 min, the supernatant was removed, and the pellets were air dried for 10 min. The pellets were then resuspended in 25 µL 0.1 M NaOH and the protein concentration was determined according to Bradford (1976) using BSA as a standard. Subsequently, equal amounts (on the basis of total protein) were loaded on 15% SDS-PAGE mini gels (Hoeffer Mighty Small II Slab Gel Electrophoresis Unit SE 250, San Fransisco, CA). Gels were run at 180 V for 1 h.

2.7. Western blots. Total protein was separated on 15% SDS-PAGE gels as above. Gels were electroblotted onto nitrocellulose membranes for 1 h at a constant 70 V according to Dunn (1986), and blocked overnight in 3% fish skin gelatin (in 1X PBS) (White and Green, 1987). Membranes were subsequently incubated with antibody directed against either cruciferin (1:500 dilution [obtained from Dr. M.L. Crouch]), oleosin (1:3000 dilution [obtained from Dr. A. Huang]), or ICL (1:100 dilution [obtained from Dr. J.J. Harada]) for 1 h at room temperature. Membranes were washed in 1X PBS/0.05% Tween 20 for 10 min three times, 1X PBS for 10 min, and incubated with Goat Anti-Rabbit IgG (Sigma) (1:3000 dilution in 3% fish skin gelatin in 1X PBS) linked to an alkaline phosphatase for cruciferin and ICL, or incubated with Goat Anti-Chicken IgG (Sigma) linked to an alkaline phosphotase for oleosin blots. Membranes were washed as above and developed according to White and Green (1987).

2.8. Data analysis. Seed was randomly collected from a population of dry seed and germinated as above. During the experiments, samples of 100 seed from each combination of DD or DAI and temperature were used. In experiments where specific developmental stages were used, seedlings of

specific radicle lengths were collected randomly. In the labelling experiment, seeds and/or seedlings representative of the average developmental stage for each combination of DAI and temperature were selected as per Table 3-1. Unless otherwise noted, all values represent the mean of at least three independent replicates ± 1 SD.

3.0. Results.

3.1. Seed germination. The use of DD (degree days) enabled us to uncouple thermal constraints from developmental impairments. By plotting the germination potential from each of the temperature conditions on the basis of equivalent heat units recieved by seed, differences in germination would then reflect differences as a result of processes involved in germination and not temporal differences in these processes. At 22°C, percent germination increased rapidly and reached the maximum by 60 DD (see Fig. 3-1). There was an initial lag at 10°C, then germination increased rapidly after 15 DD, reaching 98.7% germination by 64 DD. Germination was lower at 6°C. Again, there was a lag up to 15 DD and germination increased slowly thereafter. In order to determine which phases of germination were affected by low temperature, fresh weights of 100 seed were measured over a 24 hour period and fresh weights of germinated seed up to 160 DD. Increases in fresh weight over the first 24 hours after the onset of imbibition (HAI) are shown in Fig. 3-2. At 22°C the seed took up water rapidly, and was fully imbibed by 6 hrs. The uptake of water by the seed at 10 and 6°C was initially more gradual, but the seed was fully imbibed after 20 and 24 hrs, respectively.

3.2. Seedling growth. The fresh weight of germinated seed was calculated (change in fresh weight=[increase in fresh weight - fresh weight of fully imbibed seed] X % germination), and these values were used to measure increases only in seedling growth (Fig. 3-3). This formula shows the seedling growth of only germinated seed and therefore uncoupled the effect of processes inhibiting germinaiton in the ungerminated population. After 15 DD at 22°C, fresh weight increased gradually and seed entered phase III of germination. Thereafter, rapid increases in fresh weight were observed after 60 DD concurrent with the end of germination and the onset of seedling growth. Seed imbibed at 10°C entered phase III at approximately 30 DD, and exhibited a similar uptake in water until 64 DD, after which, fresh weight increased gradually. The rapid increase after germination

Figure 3-1. Germination percentage under various temperature conditions in *B. napus* cv. Westar on the basis of equivalent degree days (thermal time). Temperatures tested were (♦) 22°C, (■) 10°C, and (●) 6°C. Each value represents the mean of three independent replicates ± 1 SD.

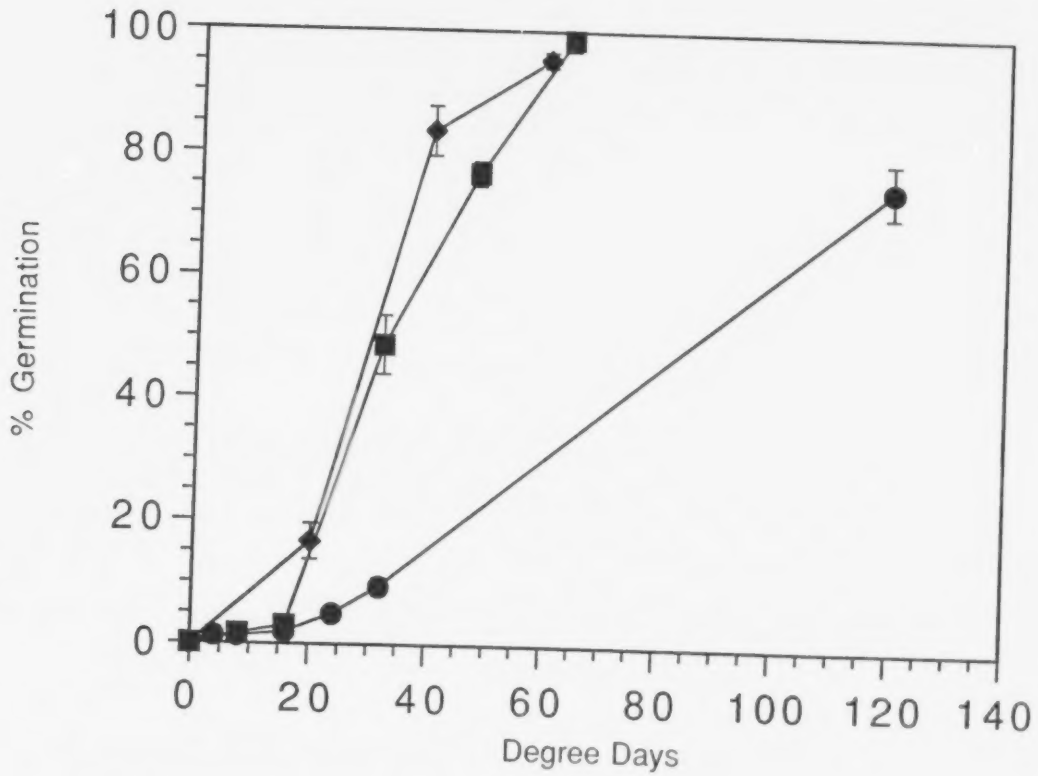


Figure 3-2. Changes in fresh weight during the first 24 hours after imbibition (HAI) under different temperature conditions in *B. napus* cv. Westar. Temperatures tested were (♦) 22°C, (■) 10°C, and (●) 6°C. Each value represents the mean of three independent replicates ± 1 SD.

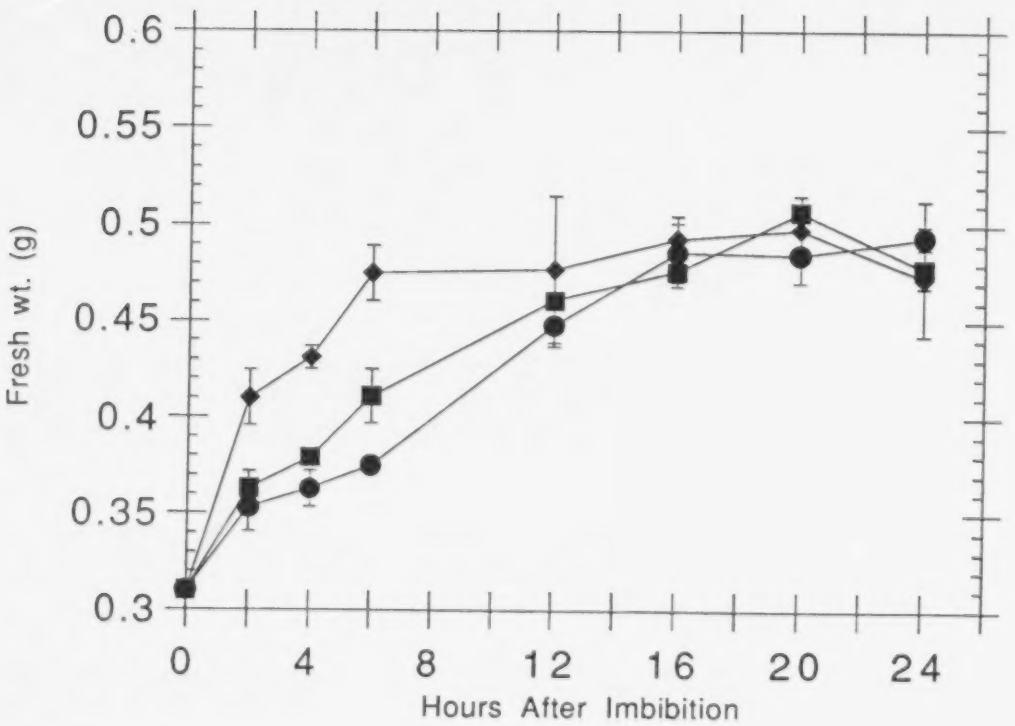
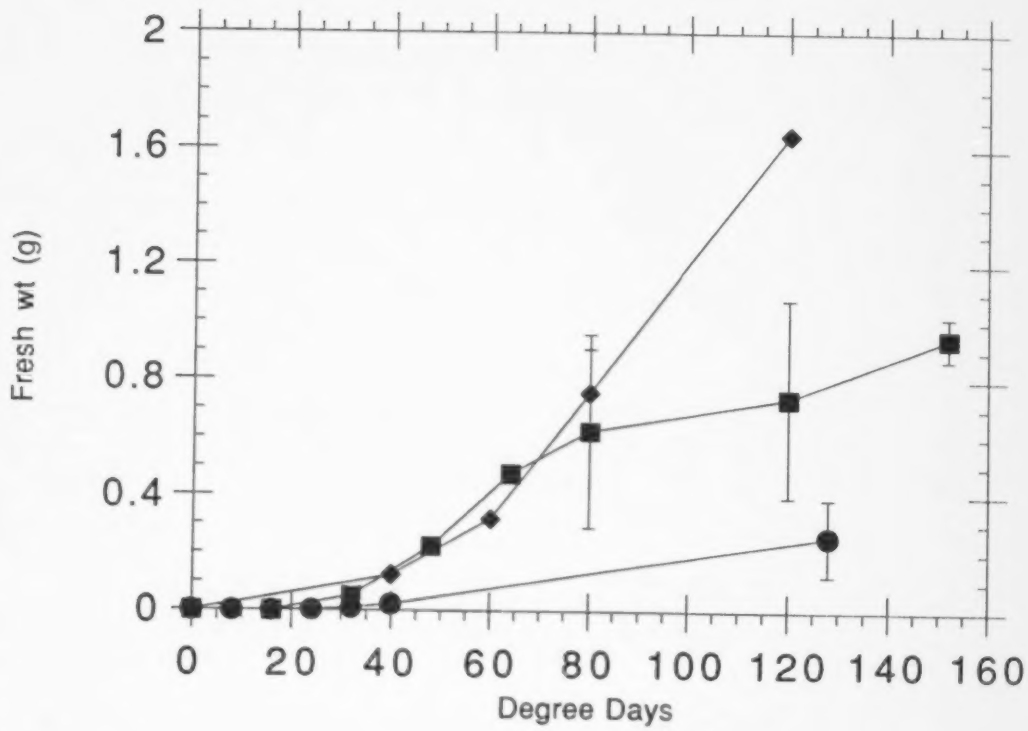


Figure 3-3. Changes in fresh weight during germination and early seedling growth under different temperature conditions in *B. napus* cv. Westar on the basis of thermal time. Temperatures tested were (♦) 22°C, (■) 10°C, and (●) 6°C. Each value represents the mean of three independent replicates ± 1 SD.



was not observed. The increase in fresh weight was low at 6°C, with phase III of germination entered after 40 DD.

Radicle lengths of germinated seed were measured and averaged to provide an index of seedling growth for each DAI. The results from this experiment are shown in Table 3-1. By interpolating values from Table 3-1 we could ascertain that the initial rate of seedling growth in germinated seed was delayed at 10°C when based on thermal time. At °6 C, seedling growth in germinated seed was delayed and lower in comparison to both the 22 and 10°C conditions.

3.3. CO_2 determinations. CO_2 levels increased steadily after imbibition at 22°C (Fig. 3-4). At 10 and 6°C CO_2 levels increased gradually over the first 16 and 8 DD, respectively. Thereafter levels remained relatively constant until 24 DD, increasing steadily afterwards in the 10°C sample. However at 6°C the levels declined back down to initial levels.

3.4. Chlorophyll accumulation. Increases in total chlorophyll occurred rapidly under optimal conditions after 40 DD (Figure 3-5). However, chlorophyll accumulation was lower at both 10 and 6°C. The 10°C sample accumulated chlorophyll after reaching 120 DD, increasing at about the same rate as the 22°C sample suggesting the onset of chlorophyll accumulation was temporally delayed. The accumulation of chlorophyll at 6°C was very low. Testing was not carried out after 120 DD, as there was little increase in fresh weight (Fig. 3-3).

3.5. *In vivo* labelling. The rate of protein synthesis indicated by methioine incorporation demonstrates storage protein degradation (Table 3-2). Samples were labelled for 40 degree hours to standardize thermal times for each temperature. The 22°C samples displayed increasing rates of protein synthesis, with high rates of methionine incorporation at 200 DD. Protein synthesis in both the 10 and 6°C samples were higher than the 22°C samples at 40 DD and at 80 DD in the 10°C sample.

3.6. Western analysis. Markers of storage reserve breakdown were studied. Polyclonal antibodies directed against cruciferin were employed

Table 3-1. Mean radicle lengths (mm) of seeds germinated at each temperature condition for days after imbibition (DAI). Mean radicle lengths were obtained as outlined in materials and methods. Degree days (DD) provide similar thermal timelines between the temperature conditions with respect to DAI. Each value represents the mean of at least three independent replicates ± 1 SD. Values in parentheses represent interpolated values calculated from the empirically measured values. Values in brackets represent DAI.

DD	22°C	[DAI]	10°C	[DAI]	6°C	[DAI]
0	0	[0]	0	[0]	0	[0]
4	(0.04)		(0.01)		0.02±0.01	[1]
8	(0.1)		0.02±0.01	[1]	0.02±0.01	[2]
16	(0.18)		0.03±0.01	[2]	0.04±0.01	[4]
20	0.22±0.1	[1]	(0.10)		(0.09)	
24	(0.60)		0.16±0.1	[3]	0.14±0.1	[6]
32	(1.35)		0.85±0.3	[4]	0.49±0.1	[8]
40	2.10±0.6	[2]	(2.10)		0.89±0.2	[10]
48	(3.31)		3.30±0.5	[6]		
60	5.13±1.0	[3]	(6.35)			
64	(5.75)		7.4±1.6	[8]		
80	8.2±0.3	[4]				

Figure 3-4. The evolution of CO_2 over the course of imbibition on the basis of equivalent thermal time in *B. napus* cv. Westar. Temperatures tested were (♦) 22°C, (■) 10°C, and (●) 6°C. Each value represents the mean ± 1 S.D. of three independent replicates.

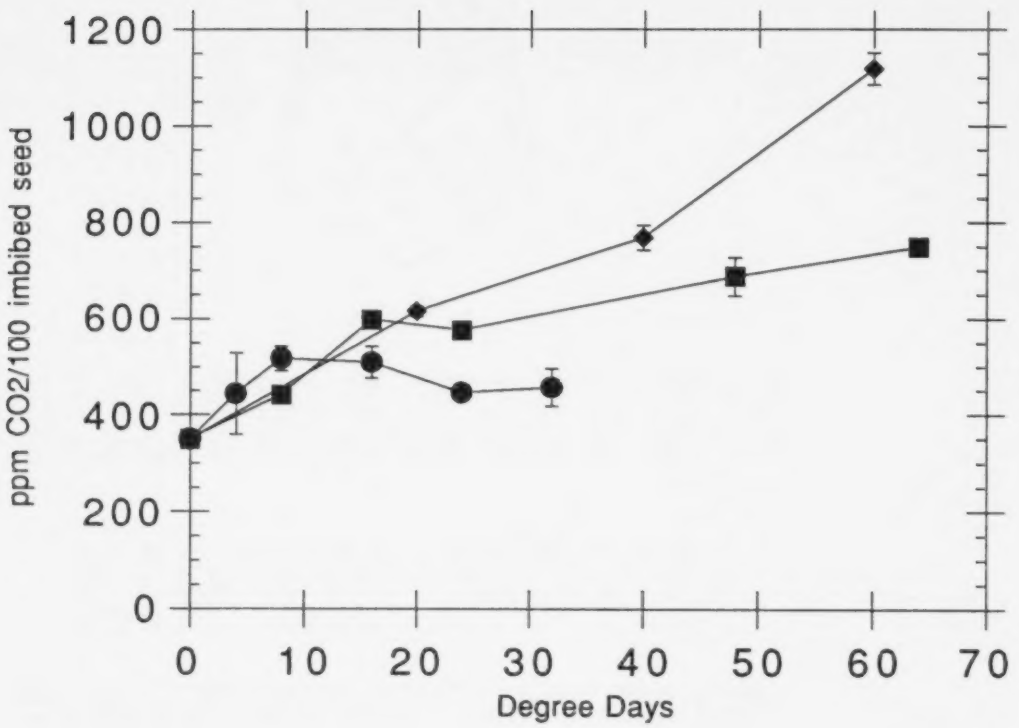


Figure 3-5. Changes in total chlorophyll during germination and early seedling growth under optimal and suboptimal temperature conditions in *B. napus* cv. Westar. Temperatures tested were (♦) 22°C, (■) 10°C, and (●) 6°C. Each value represents the mean of three independent replicates ± 1 SD.

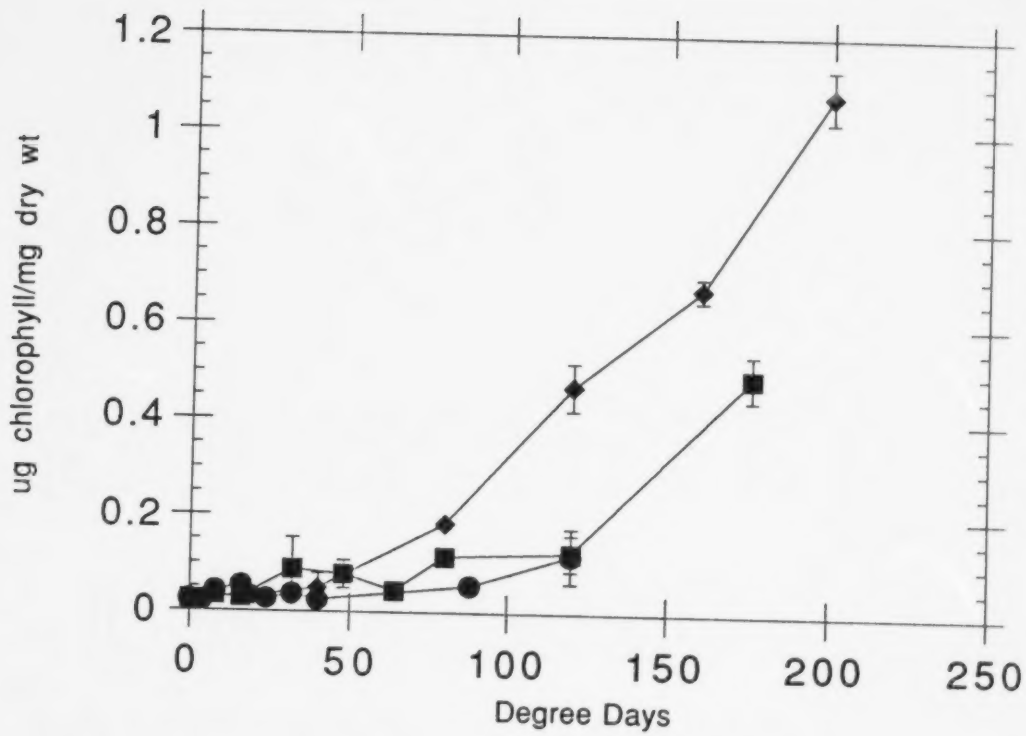


Table 3-2. Methionine (^{35}S) incorporation rates (Bqs/mg protein x 10^7) during germination and early seedling growth for optimal and suboptimal temperatures. The proteins were labelled as outlined in materials and methods. Sampling at 6°C ceased at 40 DD when it was apparent that further development was very slow. Each value represents the mean of at least three independent replicates ± 1 SD. Values in parentheses represent interpolated values calculated from empirically measured values. Values in brackets represent DAI.

DD	22°C	[DAI]	10°C	[DAI]	6°C	[DAI]
0	1.4±0.1	[0]	1.4±0.1	[0]	1.4±0.1	[0]
4	(2.5)		(1.4)		1.2±0.6	[1]
8	(3.7)		1.5±0.6	[1]	(1.4)	
16	(6.0)		(3.6)		1.7±1.2	[4]
20	7.1±1	[1]	(4.7)		(3.6)	
32	(8.9)		7.8±1.7	[4]	9.4±6	[8]
40	(10.1)		(11.6)		21.3±10	[10]
64	(13.8)		23.0±4	[8]		
80	16.2±4	[4]	31.4±6	[10]		
160	19.2±6	[8]				
200	61.5±23	[10]				

to follow its degradation over the course of postgerminative growth (Fig. 3-6). Cruciferin was not detected in the negative control (lane 1e; leaf tissue). At 22 and 10°C, cruciferin was no longer detected after the radicle had elongated 5 mm. At 6°C, cruciferin was still detected at 5 mm radicle length, but not at 10 mm. There is an initial increase in cruciferin detected for mature seed to 1mm germinated seedlings. This initial increase is believed to relect slight differences in the solubility of cruciferin between desiccated seed and germinated seedlings, and therefore this increase is the result of the methodology used and not an actual increase.

The implications of delayed protein biosynthesis at low temperature were studied by determining ICL levels. As ICL is critical in lipid mobilization this also provides insight into the effect of low temperature on lipid mobilization (Fig. 3-7). At all three temperatures, ICL increased from low levels at 1 mm radicle length to higher levels at 15 mm. However, there was more ICL detected at 22°C relative to the lower temperature conditions. Again ICL was not detected in the negative control leaf tissue (lane 1e, Fig. 3-7).

By following the breakdown of oleosin (oil body protein) we can indirectly follow the mobilization of lipids. Oleosin decreases slightly after the radicle has elongated 5 mm, but is still detected in seedlings with a 15 mm radicle in all temperature conditions (Fig. 3-8). Further analysis using seedlings from 2.0 to 3.0 cm long revealed the developmental delay in oleosin breakdown (Fig. 3-9). At 22°C, oleosin quickly diminished once epicotyl growth ensued and seedlings reached 2.5 cm. However, at 10°C oleosin breakdown occurs at 3.0 cm. There was little or no oleosin breakdown at the same developmental stage in 6°C seedlings.

Figure 3-6. Western blot analysis of cruciferin from total protein (10 µg/lane) isolated during early seedling growth (mm radicle length) under different temperature conditions in *B. napus* cv. Westar. The temperatures tested were 22, 10, and 6°C. (◂) denotes the 31 and 29 kD α-polypeptides.

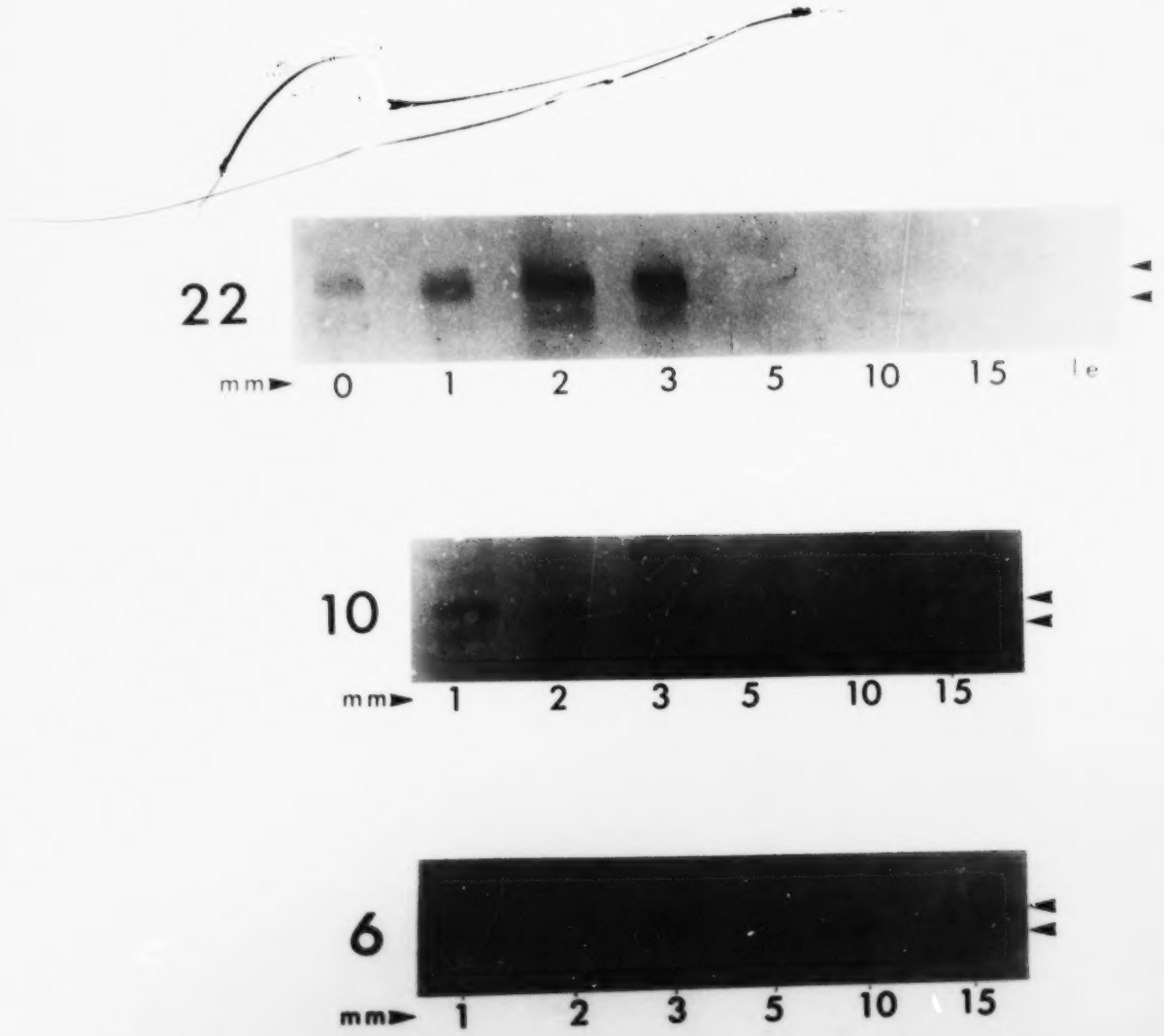

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Figure 3-7. Western blot analysis of isocitrate lyase (ICL) from total protein (10 µg/lane) isolated during early seedling growth (mm radicle length) under different temperature conditions in *B. napus* cv. Westar. The temperatures tested were 22, 10, and 6°C. (◂) denotes the 63 kD polypeptide.

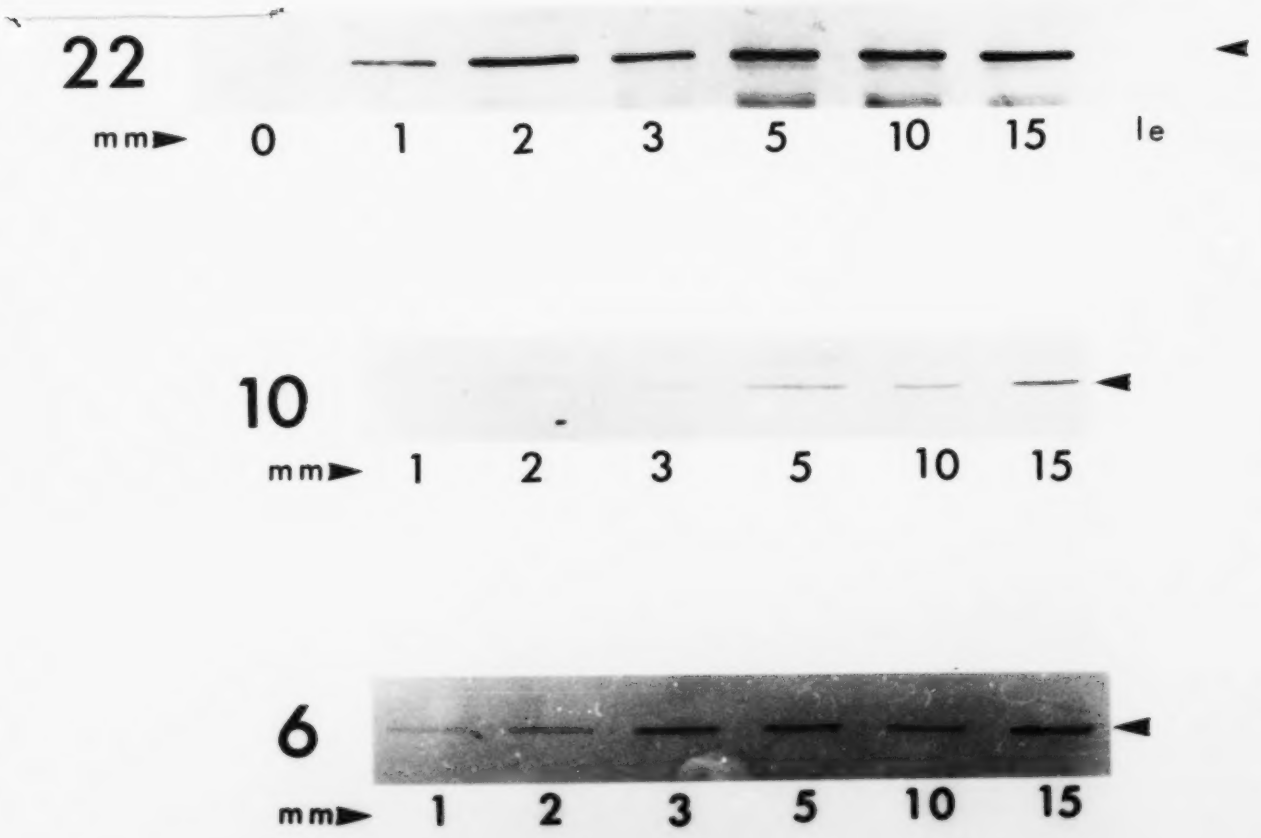
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Figure 3-8. Western blot analysis of oleosin from total protein (1 µg/lane) isolated during early seedling growth (mm radicle length) under different temperature conditions in *B. napus* cv. Westar. The temperatures tested were 22, 10, and 6°C. (◂) denotes the 20 kD polypeptide.

22
mm► 0 1 2 3 5 10 15 le
10
mm► 1 2 3 5 10 15
6
mm► 1 2 3 5 10 15

Figure 3-9. Western blot analysis of oleosin from total protein (1 µg/lane) isolated during seedling growth (mm seedlings including epicotyl growth) under different temperature conditions in *B. napus* cv. Westar. The temperatures tested were 22, 10, and 6°C. (◂) denotes the 20 kD polypeptide.

22
10
6
20 25 30 mm

4.0. Discussion.

Germination is generally reduced at temperatures below the optimum (Simon *et al.*, 1976; Liengsiri and Hellum, 1988; Mohaptra and Suggs, 1989; Steiner and Jacobsen, 1992; Leviatov *et al.*, 1994). In canola, temperatures below 10°C result in poor germination (Acharya *et al.*, 1983; Barber *et al.*, 1991), and longer germination times (Kondra *et al.*, 1983; King *et al.*, 1986; Wilson *et al.*, 1992; Mills, 1993). The temperature for baseline growth in Westar is approximately 5°C (Morrison *et al.*, 1989), and germination rarely occurs at temperatures below 2°C (Canola Growers Manual, 1991; Wilson *et al.*, 1992).

Our results are consistent with previous studies. Seeds imbibed at both 22°C and 10°C germinate rapidly and completely, requiring 60 and 64 DDs, respectively to attain the same level of germination. The temporal delay of 15 DD (equal to approximately 2 days) at 10°C is a thermal effect (Fig. 3-1). In other words, the delays observed in germination appears to result from a prolonged lag (phase II) during germination in which metabolic processes are slowed by exposure to low temperature imbibition. Thereafter, seedling growth at 10°C is initially similar to seed germinated at 22°C (Table 3-1). Differences in seedling growth become more apparent after germination is complete and seedling growth is underway (after 60 DD for 22°C and after 64 DD for 10°C in Fig. 3-3). At 6°C, germination was lower and delayed in comparison to both the 22 and 10°C conditions. The reduction in germination at 6°C not only reflects temporal delays in processes involved in germination , but also suggests that these processes are somewhat impaired at this temperature. Furthermore, seedling growth was also reduced (Fig. 3-3).

We were interested in determining which phase of germination was affected by low temperature. In a related crucifer, *Sinapis alba*, low temperature increased phase II, reduced radicle growth, and led to lower respiration rates (Simon *et al.*, 1976). There was a slight lag in water uptake during phase I (Fig. 3-2) at 10 and 6°C. This has been observed in

soybean (Vertucci and Leopold, 1983; Vertucci, 1989), and is expected to damage the embryo (Simon *et al.*, 1976; Bradbeer, 1988; Bewley and Black, 1994). In the present study, the delay did not damage the seed at 10°C, but may have at 6°C as indicated by a prolonged phase II, reduced radicle growth, and lower respiration rates.

The transition from phase II to phase III has been shown to be most sensitive to temperature (Simon *et al.*, 1976). During this period, metabolic processes such as respiration (Simon, 1984), protein synthesis (Bewley and Black, 1994), ATP production (Mayer and Marbach, 1981; Simon, 1984), and RNA synthesis (Payne *et al.*, 1978; Lane, 1991) are occurring leading up to end of germination after phase III. The delay observed in germination in the present study appeared to result from a prolonged phase II (Fig. 3-2, Fig. 3-3). At 10°C the transition was delayed relative to the 22°C temperature condition. At 6°C, the seed entered phase III gradually after a prolonged stasis in phase II (Fig. 3-3), resulting in low rates of germination and low increases in fresh weight.

In canola, seedling growth has been assessed with respect to different genotypes (cultivars) (Acharya *et al.*, 1983; King *et al.*, 1986) and seedlots (Barber *et al.*, 1991). These studies reveal low temperature reduces seedling growth and subsequent emergence. The seedling index (Table 3-1) shows rapid growth in germinated seed at 22°C and 10°C, and low seedling growth at 6°C. The reduction in seedling growth was corroborated by differences in fresh weight increases (see Fig. 3-3). Whereas seedling growth was rapid at 22°C, it was delayed and lower at 10°C, and further delayed and reduced at 6°C. Thus, in the present study, both germination and early seedling growth were affected by reduced growth temperatures. Our results indicate that low temperature has two effects: a temporal delay at 10°C and reduced seedling growth thereafter, and a delay and reduction in both germination and seedling growth at 6°C.

Associated with lipid mobilization is the evolution of CO_2 (Mathews and Van Holde, 1990). The respiration rates displayed by seed at 22°C are

indicative of the processes involved in post germinative growth (i.e. carbohydrate and lipid mobilization). At 10 and 6°C there is an initial increase in CO_2. This initial rise is most likely the result of stored carbohydrate (upto 10% total seed weight constitutes amylose and amylopectin in *B. napus*) mobilization. At 10°C we observe a steady increase in CO_2 levels after 24 DD, which is concurrent with increases in germination. At 6°C the levels return down to initial rates (approximately 450 ppm/100 seed).

Chlorophyll is an essential part of the photosynthetic machinery that enables a plant to attain autotrophic growth. At low temperature, there is a temporal lag in chlorophyll accumulation in etiolated rye seedlings when placed under illumination compared to seedlings grown at the optimum temperature (Krol and Huner, 1984; Krol *et al.*, 1987). In the present study, we observed a reduction in chlorophyll accumulation at the suboptimal temperatures. The 10°C seedlings accumulate chlorophyll after a temporal delay of 120 DD. However, at 6°C, the lack of chlorophyll may suggest that the photosynthetic machinery cannot be assembled, and therefore post-germinative growth is inhibited. The observed increases in chlorophyll correlated well with the increases in fresh weight for the 22°C sample (compare Figures 3-3 and 3-5). At 10°C the increases in fresh weight were not accompanied by similar increases in chlorophyll, and this may be a contributing factor in the observed reduction in seedling growth. At 6°C there were both low levels of chlorophyll accumulation and low increases in fresh weight, again indicating poor seedling growth.

Low temperature has been shown to alter protein synthesis (Meza-Basso *et al.*, 1986; Johnson-Flanagan *et al.*, 1991). In the present study protein synthesis does not appear to be impaired, but is temporally delayed (Table 3-2). The length of the delay is directly related to the temperature; ie. the lower the temperature the longer the delay. This suggests that protein synthesis and/or deamination followed by transamination is developmentally delayed at lower temperatures.

Therefore, processes prior to *de novo* protein synthesis may also be delayed. Moreover, these processes are only delayed because of thermal constraints, as indicated by the DD comparisons.

Developmentally similar seedlings should display comparable rates of protein mobilization, unless there is a loss of coordination of storage reserve breakdown. This was examined by following storage reserve mobilization (Fig. 3-6). Our results indicate that protein mobilization at 10°C is delayed because of thermal constraints, whereas at 6°C there is a loss of coordination possibly because of lower rates of storage protein hydrolysis (compare Fig. 3-6 to Fig. A-1 in the appendix).

After germination, storage proteins are degraded by endo- and exopeptidases (Murray, 1984; Bewley and Black, 1994). According to Bhandari and Chitralekha (1989), the initiation of protein degradation in *B. campestris* is simply triggered by imbibition. The current study shows that imbibition alone is not enough to initiate cruciferin mobilization at low temperature. Seed that germinated at 6°C, and thereby entered phase III of germination, exhibited low rates of protein mobilization (Fig. 3-6).

ICL is normally considered to be a good indicator of the switch from germination to seedling growth (Harada *et al.*, 1988) in addition to being indicative of lipid breakdown (Trelease, 1984). In a previous study we observed lower lipid depletion and ICL activity at 10 and 6°C (Figures 2-4 and 2-3; chapter 2). In the present study, lower ICL levels were observed at the lower temperatures in comparison to the 22°C sample (Fig. 3-7). Therefore, the lower amount of ICL detected in samples from the lower temperatures suggest that a lack of enzyme biosynthesis, rather than reduced activity results in poor lipid mobilization, as previously measured. Thus, ICL biosynthesis may contribute to lower seedling growth at 10°C, and very poor seedling growth at 6°C.

Another marker of lipid mobilization is the degradation of oleosin during early seedling growth. The present study reveals that the

breakdown of oleosin appears to be similar at 22, 10, and 6°C during early seedling growth. Therefore, processes associated with the initial breakdown of oil bodies, i.e. lipases, and subsequent breakdown of oleosins do not appear to be impaired. However, once epicotyl growth begins, developmental delays were observed at 10 and 6°C (Fig. 3-9).

Results from the present study indicate that reduced seedling emergence at low temperature is a function of both germination and early seedling growth. At 10°C, germination rates slow without reducing the germination success. This appears to be strictly a thermodynamic effect. On the other hand, there is a large reduction in seedling growth. The reduced seedling growth as measured by fresh weight is associated with lower rates of CO_2 evolution, lower rates of chlorophyll accumulation, lower rates of ICL synthesis, developmental delays in oleosin depletion, and temporal, but not thermodynamic delays in protein synthesis. This does not appear to be caused by decreased storage protein degradation. The low rate of fresh weight increase may indicate a developmental effect. Seed at 6°C showed poor germination and poor seedling growth. A proportion of the seed is unable to make the transition from phase II to phase III. Further, seedling growth was also affected by a loss of developmental co-ordination, i.e. a loss of coordination between the breakdown of storage reserves (cruciferin; and lipids via ICL synthesis and oleosin degradation) and their subsequent utilization. We speculate the lack of coordination between the mobilization of storage proteins and the *de novo* synthesis of proteins (compare Fig. 3-6 and Table 3-1) prevents some seed from germinating and proceeding with growth at 6°C.

5.0. References.

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Chapter 4. Gene Expression During Germination and Early Seedling Growth Under Low Temperature Conditions in *Brassica napus* cv. Westar.

1.0. Introduction.

During early sporophytic growth a number of developmental stages have been delineated. In general terms these are designated embryogenesis, germination and early seedling growth. In *B. napus*, these have been further divided into specific temporal stages that are related to molecular, biochemical, and morphological changes.

Embryogenesis, the period encompassing the formation of the zygote until the development of the mature dry seed, has been described in detail by a number of researchers (Tykarska, 1976, 1979, 1980, 1987; Crouch and Sussex, 1981; Fernandez *et al.*, 1991; West and Harada, 1993; Jakobsen *et al.*, 1994). After quiescence there is a developmental switch in the programs from embryogenesis to germination (Harada *et al.*, 1988; Kermode, 1990) initiated by imbibition. Germination is defined as the developmental period beginning with water uptake and ends with radicle emergence through the seed coat (Bewley and Black, 1994). This period has also been studied (Kuras, 1984, 1986, 1987; Harada *et al.*, 1988). Following germination there is another developmental switch to processes associated with early seedling growth that lead to autotrophic growth and development (Harada *et al.*, 1988; Dietrich *et al.*, 1989).

Numerous studies have examined gene expression during late embryogenesis (35-60 DAF) and early seedling growth (Harada *et al.*, 1988; Dietrich *et al.*, 1989; Fernandez *et al.*, 1991; Jakobsen *et al.*, 1994). By following molecular messages throughout these stages, the timing of the developmental switches and possible mediators of the switches have been elucidated.

During the later stages of embryogenesis, mRNAs fall into one of two classes; residual and conserved mRNAs (Kermode, 1990; Bewley and Black, 1994). Residual mRNAs are produced during seed development, persist through seed maturation and desiccation, and are not necessary to the germination process and may be degraded upon imbibition. Conserved or stored mRNAs are produced during seed development, are available for

translation upon hydration, and are an integral part of germination.

One class of residual mRNAs, derived from the late embryogenesis-abundant (*Lea*) genes (Galau *et al.*, 1986), have been characterized. *Lea* type messages are highly conserved across species including cotton (Baker *et al.*, 1988), carrot (Choi *et al.*, 1987), barley (Hong *et al.*, 1988), and rice (Mundy and Chua, 1988). In *B. napus*, a number of *Lea* messages have been temporally characterized (Harada *et al.*, 1989; Raynal *et al.*, 1989; Jakobsen *et al.*, 1994). During late embryogenesis LEA 76 messages increase in abundance (26-35 DAF), persist at high levels during desiccation (38-42 DAF), are present at high levels in the mature seed (60 DAF), and are degraded rapidly upon imbibition (absent after 16-24 HAI) (Harada *et al.*, 1989). Therefore, the loss of these messages signals the end of embryogenesis and the onset of germination.

The conserved or stored mRNAs are activated during late embryogenesis and are induced during germination and early seedling growth (Harada *et al.*, 1988; Goldberg *et al.*, 1989). In *B. napus*, two members belonging to this class are COT44 and ICL (Harada *et al.*, 1988; Dietrich *et al.*, 1989; Ettinger and Harada, 1990). COT 44 is present in low amounts during late embryogeny, increases during the first 24 HAI, and accumulates to high levels during post-germination in the seedling (Harada *et al.*, 1988; Dietrich *et al.*, 1989). In a similar manner, ICL message is present during late embryogeny (35-40 DPA), and increases dramatically to high levels over the first 6 days of imbibition (Comai *et al.*, 1989). These messages, therefore, signal the onset of germination and post-germinative growth.

ABA has been implicated as a mediator of the developmental switches (Kermode, 1990; Sing and Sawhney, 1992). For example, ABA has been shown to prevent precocious germination in immature rapeseed embryos (Finkelstein *et al.*, 1985). However, endogenous ABA levels are low in the mature dry seed of *B. napus* (Finkelstein *et al.*, 1985; Kermode, 1990; Singh and Sawhney, 1992). In *Arabidopsis* ABA deficient mutants, seed

germination was promoted (Karssen *et al.*, 1983). Furthermore, ABA has been demonstrated to inhibit and reduce germination (Schopfer and Plachy, 1984; Schopfer and Plachy, 1985; Zeevaart and Creelman, 1988; Sing and Sawhney, 1992; Sharma *et al.*, 1992). The effect of ABA during seed germination has not been empirically defined, but hypotheses include the inhibition of water uptake during imbibition (Schopfer *et al.*, 1979; Schopfer and Plachy, 1984) by preventing cell wall extension (Schopfer and Plachy, 1985). There has been little exploration on the effect(s) of ABA on seed germination in canola, and no studies with relation to low temperature germination. Dr. Wilen (personal communication) demonstrated increases in low temperature germination using ABA antilogs developed by Dr. Susan Abrams at the Plant Biotechnology Institute in Saskatoon. Therefore, an ABA synthesis inhibitor, fluridone (Gamble and Mullet, 1986) in theory should alleviate the effects of any endogenous ABA accumulation via biosynthesis on seed germination at optimal and suboptimal temperatures.

ABA has been implicated as a mediator of the developmental switches (Kermode, 1990). *Lea* messages are upregulated by exogenous ABA (Galau *et al.*, 1991; Hughes and Galau, 1991; Jakobsen *et al.*, 1994). Messages encoding ICL, however, were not affected by exogenous ABA (Jakobsen *et al.*, 1994). Other germination specific cDNAs from cotton (Hughes and Galau, 1991) encoding rubisco subunits (*RbcS*) and chlorophyll a/b binding protein *(Cab)*, both which can be classified as post-germination specific messages, were observed to decrease in the presence of ABA in cultured embryos (Jakobsen *et* al., 1994). Bisgrove *et al.* (1995) demonstrated that the expression of LEA 76 and COT 1 (Harada *et al.*, 1988; 1989) is sequential in excised zygotic embryos. These results lend validity to the hypothesis that embryogenesis, germination and post-germinative events are mutually exclusive, occurring sequentially, with developmental switches in place (Kermode, 1990).

Previous to the present study we documented processes associated

with low temperature germination and early seedling growth (Chapters 2 and 3). From these studies we demonstrated that seedling emergence is a function of both germination and seedling growth at low temperature. The delays in germination appear to result from thermodynamic constraints, while reduced and delayed seedling growth are a combination of both thermodynamic constraints and developmental impairment. These studies suggest that the switch in developmental programs from embryogenesis to germination and subsequent early seedling growth may be compromised by imbibition and growth at low temperature. The present study examines the effect of low temperature on the developmental switches from embryogenesis to germination and from germination to post-germinative growth. In addition, further insight into the coordination of storage reserve mobilization was gained.

2.0. Materials and methods.

2.1. Plant material. The plant material used in the present study is the same *B. napus* cv. Westar seed used in chapter 3. In addition, *B. napus* cv. Westar seed was collected from the University of Alberta Farm for low temperature germination in the presence of ABA, fluridone, or in the absence of any exogenous factors to test the hypothesis of whether endogenous ABA levels delay or reduce germination under low temperature conditions.

2.2. Seed germination. Seeds were germinated at 22, 10, 6, and 2°C as described previously (see chapter 3). Entire 100 seed set samples were used for Western blot and Northern blot analysis. In Northern blots displaying seedling developmental stages, selected seedlings (20-30) of specific radicle length were used. For ABA analysis, random sets of eight seeds were collected from freeze dried populations (100 seed set).

2.3. RNA extraction. This procedure has been modified from Verwoerd *et al.* (1989). Plant material (100 seed for DAI and 20-30 seedlings for specific developmental stages) was frozen in liquid nitrogen in a mortar on ice. The tissue was ground with a small pestle while maintained on ice. Motars and pestles were baked at 250°C for four hours and all glassware used for RNA extraction and analysis was DEPC-treated and autoclaved prior to use. While grinding, 1 mL hot (80°C) extraction buffer was added [phenol: 0.1 M LiCl, 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS (1:1)]. The slurry (500 µL) was transfered to a 1.5 mL Eppendorf tube and the mixtures were homogenized by repeated vortexing for 20 seconds each (3X), and placed on ice. Next, 250µL chloroform:isoamylalcohol (24:1) was added, and the mixture vortexed for an additional 30 secs. Samples were microfuged for 5 min at 13 000 rpm. The aqueous phase was removed and placed in a clean sterile tube. To the aqueous phase one volume of 4 M LiCl was added and the RNAs were allowed to precipitate at -20°C overnight. RNAs were pelleted by microfuging the samples for 15 min at 13 000 rpm. The supernatant was poured off and the pellet resuspended in 250 µL of

DEPC-treated water. The RNAs were then ethanol precipitated with the addition of 0.1 volume 3 M NaOAc (pH 5.2) and two volumes of cold (-20°C) ethanol. The RNAs were allowed to precipitate on ice for 1 hr. The RNAs were pelleted by microfuging for 10-15 min at 13 000 rpm. The pellet was then washed with 70% cold ethanol and microfuged again for an additional 5 min. The ethanol was poured off and the pellet was dried in a speed vac. The dried pellet was then resuspended in DEPC-treated water (50-100 µL) and quantified by spectrofluorimetry according to the method of Harris (1987).

2.4. Northern blotting; gel preparation and electrophoresis conditions. The methods of northern blotting were adapted from Fourney *et al.* (1988). 20 µg of total RNA was seperated on a formaldehyde denaturing gel. The gel was prepared as follows: 1.25 g of agarose (ultra pure; Bio-Rad) was dissolved in 1X MOPS buffer in DEPC-treated ddH_2O in an RNase-free flask, the agarose solution was allowed to cool to 50°C, thereafter 5.1 mL 37% formaldehyde was added and the agarose solution was gently mixed. Finally, the solution was poured into a 14x20-cm gel tray. The gel was allowed to sit in a fume hood for 1 hour prior to use. Quantified RNA samples were lyophilized, dissolved in 5 µL DEPC-treated ddH_2O, and denatured at 65°C for 15 min after the addition of 30 µL electrophoresis sample buffer (0.75 mL deionized formamide, 0.15 mL 10X MOPS, 0.24 mL formaldehyde, 0.1 mL DEPC-treated ddH_2O, 0.1 mL glycerol, and 0.08 mL 10% [w/v] bromophenol blue). After samples were denatured they were returned to ice and 1 µL ethidium bromide (1mg/mL) was added. Samples were then separated on formaldehyde denaturing gel at 200 V, for approximately 1-2 hours, in electrophesis buffer (1X MOPS).

Separated RNA was visualized on a UV transilluminator to ensure equal loading and separation occurred. In addition, 10 ug of total RNA from the DAI and stage specific samples was slot blotted onto Zeta-Probe membrane (Sigma) and tested for equal loading with a cDNA ribosomal probe

(pMF2 from Dr. K. Eggar). Thereafter total RNA was transferred onto Zeta-Probe membrane (Sigma) via capillary action, followed by crosslinking to the membrane by UV light (254 nm) for 2 min at 30 cm. Gel was prepared for transfer by soaking in 10XSSC for 2X 20-min periods, concurrently Zeta-Probe (Sigma) membrane was soaked 5 min in DEPC-treated ddH_2O followed by a 10-min soak in 10XSSC. The transfer took place in RNase-treated glassware, in 10XSSC buffer, and capillary action was allowed to proceed for 16 hours.

2.5. Plasmid isolation. Plasmids containing pLEA 76 (Harada *et al.*, 1989), pB86 (Raynal *et al.*, 1989), pIL1 (Dietrich *et al.*, 1989), and pCOT44 (Harada *et al.*, 1988) were amplified on a large scale basis separately in LB with 12.5 µg/mL tetracycline for selective resistance (Maniatis, 1981). Plasmids were then isolated according to a modified procedure of Sambrook *et al.* (1989). Large scale plasmid preparations (1L cultures) were pelletted from overnight cultures at 5000 g for 5 min at 4°C in GSA-600 rotor (Sorvall). LB supernatant was poured off and cells were suspended in cold (4°C) 100 mL STE buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA). All subsequent operations were carried out on ice. Cells were pelleted in GSA rotor (Sorvall) (5000 g for 5 min at 4°C). Supernatant was decanted and the cells were resuspended in 20 mL in 10% sucrose buffer (10% sucrose, 50 mM Tris-HCl pH 8.0 at 4°C). Cell lysis was carried out with the addition of 4 ml of a freshly prepared solution of lysozyme (10 mg/mL lysozyme in 0.25 M Tris-HCl pH 8.0) and 13.3 mL of 0.3 M Na_2EDTA pH 8.0. Samples were mixed by inversion and allowed to sit on ice for 10 min. Next, 8 ml of 10% SDS was added, samples mixed by inversion, 12 mL of 5 M NaCl was added, and samples were re-mixed by inversion. Samples were returned to ice for 1 hour with occasional mixing. Samples were then transfered to polycarbonate tubes and centrifuged in an SS-34 rotor (Sorvall) at 18 000 g for 30 min at 4°C to remove high molecular weight DNA. The supernatant was decanted to a clean polycarbonate tube. 0.6 volumes of cold isopropanol was added and samples

were mixed well and place on ice for 10 min. Nucleic acids were pelleted by centrifugation in an SS-34 rotor (Sorvall) at 12 000 g for 10 min at 4°C. The supernatant was decanted and the pellets were resuspended in 7 mL of cold ddH_2O. Insoluble material was removed by centrifugation in an SS-34 rotor (Sorvall) at 10 000 g for 5 min at 4°C. The supernatant was decanted into clean 15 mL Corex tubes. CsCl (1 g/mL) and 600 µL ethidium bromide (10 mg/mL) was added and samples placed in the dark for 15 min at room temperature. Proteins and insoluble material were removed by centrifugation at 15 000 g for 15 min at 4°C in a SS-34 rotor (Sorvall). The supernatant was transferred to a clean Corex tube and 500 µL of ethidium bromide was added (10mg/mL). Samples were transferred to VTi65 quick-seal tubes and sealed. Samples were then centrifuged for 6 hours at 54 000 g in the VTi65 rotor at room temperature using an ultracentrifuge.

Supercoiled DNA (lower band) was visualized by a UV transilluminator and collected with a syringe. The ethidium bromide was extracted with salt-saturated isopropanol and dialyzed against ddH_2O to remove CsCl. Thereafter, DNA was precipitated with 1/2 volume of 7.5 M NH_4Ac pH 7.5 and 2 volumes of cold 95% ethanol for 1 hr at -20°C. Plasmid DNA was microfuged for 15 min at 13 000 rpm. DNA pellets were washed twice with 70% ethanol and dried on speed vac for 1 hour. Samples were then resuspended in ddH_2O (50-100 µL), quantified on spectrophotometer (1 A_{260}=50 µg/mL of DNA), and aliquots digested with the restriction enzyme PstI. 2 µg of plasmid DNA was digested and fragment sizes were confirmed on agarose gels.

2.6. Labelling of isolated cDNA fragments. cDNAs were isolated by cutting separated fragments from agarose gels and purifying on Spin-X columns (Costar). Thereafter, aliquots were collected from Spin-X (Costar) columns and ethanol precipitated with 1/10 volume 3.0 M NH_4Ac pH 5.2 and 2 volumes cold 95% ethanol. cDNAs were pelleted, washed twice with 70% ethanol, and dried on a speed vac. Samples were then resuspended

in 10 µL ddH_2O and quantified on a spectrophotometer. Thereafter 50 ng of cDNA was radioactively labelled with a random primer labelling kit (Amersham) with $dCTP^{32}$ (Amersham). The activities of the probes used for northern analysis were as follows: LEA 76, 7.76×10^8 CPM/µg DNA; B86, 1.32×10^8 CPM/µg DNA; IL1, 5.61×10^8 CPM/µg DNA; COT 44, 2.64×10^8 CPM/µg DNA.

2.7. Hybridizing procedure and conditions. The hybidizing procedure was carried out according to the methods outlined in the Zeta-probe blotting membrane instruction manual (Bio-Rad). Prehybridization took place in heat sealable plastic bags in the following solution: 50% deionized formamide, 0.25 M $NaHPO_4$ pH 7.2, 0.25 M NaCL, 7% (w/v) SDS, and 1 mM EDTA. Membranes were prehybridized at 43°C for 1 hour. Hybridization took place in the same solution with the addition of the labelled probe (as outlined above), simply by cutting a corner of a bag and directly placing the radio-labelled probe into the solution. Bags were then resealed and incubated at 43°C overnight. Membranes were washed the following day briefly in 2 X SSC followed by 15 min washes in 2 X SSC/0.1% SDS at room temperature with agitation, 0.5 X SSC/0.1% SDS at room temperature with agitation, and 0.1 X SSC/0.1% SDS at 65°C. Membranes were then exposed for various periods of time in x-ray cassettes with intensifying screens and developed. Autoradiograms were developed by washing in GBX (Kodak) developing solution for 90 sec, followed by 15 sec in stopbath (Kodak), and fixed in GBX (Kodak) fixing solution for 3 min. Films were then washed in water for 10 min and air dried. Membranes were stripped by washing 2 times, 20 min each, in a large volume of 0.1 X SSC/0.5 % SDS at 95°C. To ensure membranes were clean they were subjected to overnight exposure and x-ray development as previously described. Clean membranes were then re-prehybridized and reprobed with a different cDNA.

2.8. SDS-PAGE. Seed and seedlings were collected from each temperature condition on the basis of DAI (100 seeds for each test). Sample preparation, quantification, and electrophoresis running conditions were

the same as the procedures outlined in chapter 3.

2.9. Western blots. Total protein was separated on 15% SDS-PAGE gels as above. Electroblotting of the gels onto nitrocellulose membranes, antibody incubation conditions, and subsequent ICL was carried out according to the protocol outlined in chapter 3.

2.10. ABA Analysis. Samples were collected (100 seeds) for each DAI from each temperature conditions. Samples were placed in liquid nitrogen then lyophilized in the dark. Thereafter, eight seeds were collected, weighed, and ground in liquid nitrogen in the presence of acid washed sea sand. The samples were further ground in extracting methanol (methanol containing 100 mg/L butylated hydroxytoluene and 0.5 g/L citric acid monohydrate) at a ratio of 0.01 g dry tissue: 1.0 mL extraction methanol. Extracts were covered in tin foil and stirred overnight at 4°C on a rotary shaker. After 12 hours the samples were centrifuged at 2000 g for 10 min and the supernatants collected in clean tubes. Supernatants were dried on a Speed Vac. Dried samples were resuspended in 100µL methanol and 900µL TBS. Samples were maintained in the dark as much as possible throughout the entire procedure to prevent photodegradation. From this point on the samples were analyzed for ABA according to the procedures outlined in the abscisic acid immunoassay detection kit (Sigma). The ELISA method employed in the present study has been demonstrated to be as sensitive to quantification of endogenous ABA levels as HPLC methods (Walker-Simmons, 1987). Endogenous ABA levels were obtained by comparing samples to prepared standards of isomeric ABA (Sigma) (0, 0.05, 0.1, 0.5, 2.0, and 100.0 picomole standards).

Endogenous ABA levels were assessed on the basis of fresh weight. The increases in dry weight during germination and early seedling growth are minimal (Bewley and Black, 1985) and most increase in weight is attained through the uptake in water during cell expansion. Therefore, any changes in ABA concentration would be in relation to the amount of solvent present in the cells. This is important because if ABA does not

change during germination or early seedling growth, then the uptake of water would result in a dilution effect.

2.11. Germination in the presence of ABA or fluridone. Westar seed was germinated at 22, 10, and 6°C as previously described (Chapter 3). The seed was surface sterilized by soaking for 5 min in 6% bleach solution followed by repeated rinses in ddH_2O. Replicates were also germinated in the same manner in the presence of 1 and 10 µM/L ABA, or in the presence of 1 and 10µM/L fluridone (1-methyl-3-phenyl-5-[3-(triflurомethyl)phenyl]-4(1H)-pyridinone). ABA was delivered to flasks from a 1 mM stock solution. ABA (13.215 mg) was dissolved in 100µL 0.5 M KOH, and the total volume was brought upto 5 mL in ddH_2O. The solution was heated at 75°C for 10 min and the solution was filter sterilized with a 0.2 µm Nalgene syringe filter into a sterile Falcon tube. Fluridone was delivered to flasks from a 10 mM stock solution. Fluridone (32.932 mg) was dissolved in 1 L ddH_2O with gentle stirring overnight under low heat. The solution was stirred and subsequently stored in the dark to prevent photodegradation.

3.0. Results.

3.1. Seed germination. Seed germination was remeasured in the present study to produce consistent and reliable results with respect to the present experiments. At 22°C germination percentage increased rapidly and reached the maximum by day 3 (Fig. 4-1). In relation, germination was lower in the suboptimal temperatures. At 10°C germination increased gradually until day 2, then increased rapidly 4 DAI reaching 95% germination by day 8. Germination was low at 6°C, increasing slowly over the 8 day test. In comparison seed imbibed at 2°C exhibited no germination.

3.2. Late Embryogenesis Abundant (LEA) Transcripts. The switch from embryogeny to germination was followed with two LEA type cDNAs. Over the course of imbibition (DAI) LEA 76 disappeared rapidly in the 22°C samples and was absent by 1 DAI (Fig 4-2). At 10°C, the LEA 76 message was low on 1 DAI, but then increased until 6 DAI. Thereafter LEA 76 decreased back to low levels at 8 DAI. At 6°C the message decreased 1 to 2 DAI and then increased slightly before decreasing again on 8 DAI.

At specific developmental stages, LEA 76 was absent in the 22°C tissue as early as 1 mm radicle length (Fig. 4-3). However, small amounts of transcript were still detected in the 10 and 6°C samples at 1 mm radicle length under the same hybridizing conditions, but were gone thereafter. These results have been repeated with the same observations obtained. The same is true for all observations obtained with the DAI and specific developmentally staged material for all probes used, and therefore the results obtained are valid and reliable.

At 22°C, B86 was undetected after 2 DAI (Fig. 4-2). At 10°C, B86 did not decrease until after 2 DAI. Thereafter, B86 decreased from 4 to 8 DAI. At 6°C, B86 was present in large amounts until 6 DAI when there was a noticeable decrease.

At specific developmental stages, B86 was only detected in the dry seed and was undetectable by 1 mm radicle length in all temperature

Figure 4-1. Germination percentage under the optimal and suboptimal temperature conditions in *B. napus* cv. Westar over the course of imbibition. Temperatures tested were (♦) 22°C, (■) 10°C, (●) 6°C, and (▲) 2°C. Each value represents the mean of three independent replicates ± 1 SD.

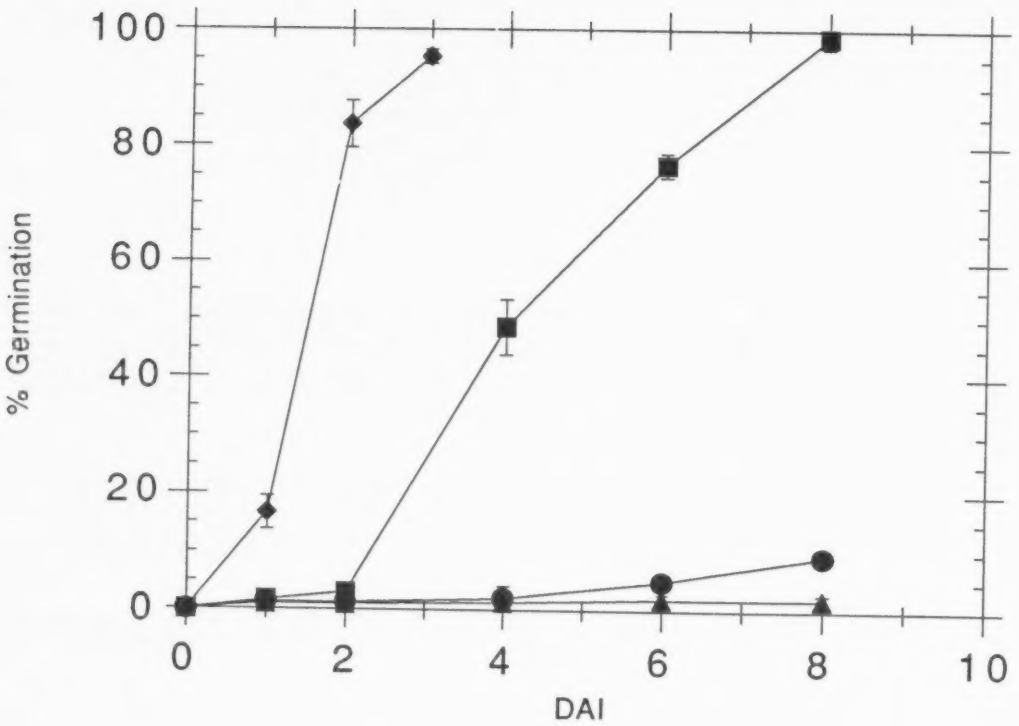


Figure 4-2. Northern blot analysis of the developmental molecular markers LEA 76, B86, IL1, and COT 44 over the course of imbibition at 22, 10, and 6°C. Each lane represents the total extracted RNA (20µg) from one hundred seed collected on each DAI in each temperature condition from *B. napus* cv. Westar seed. RNA extraction, blotting, and hybridization conditions are outlined in the material and methods section. Sd denotes total RNA extracted from mature seed.

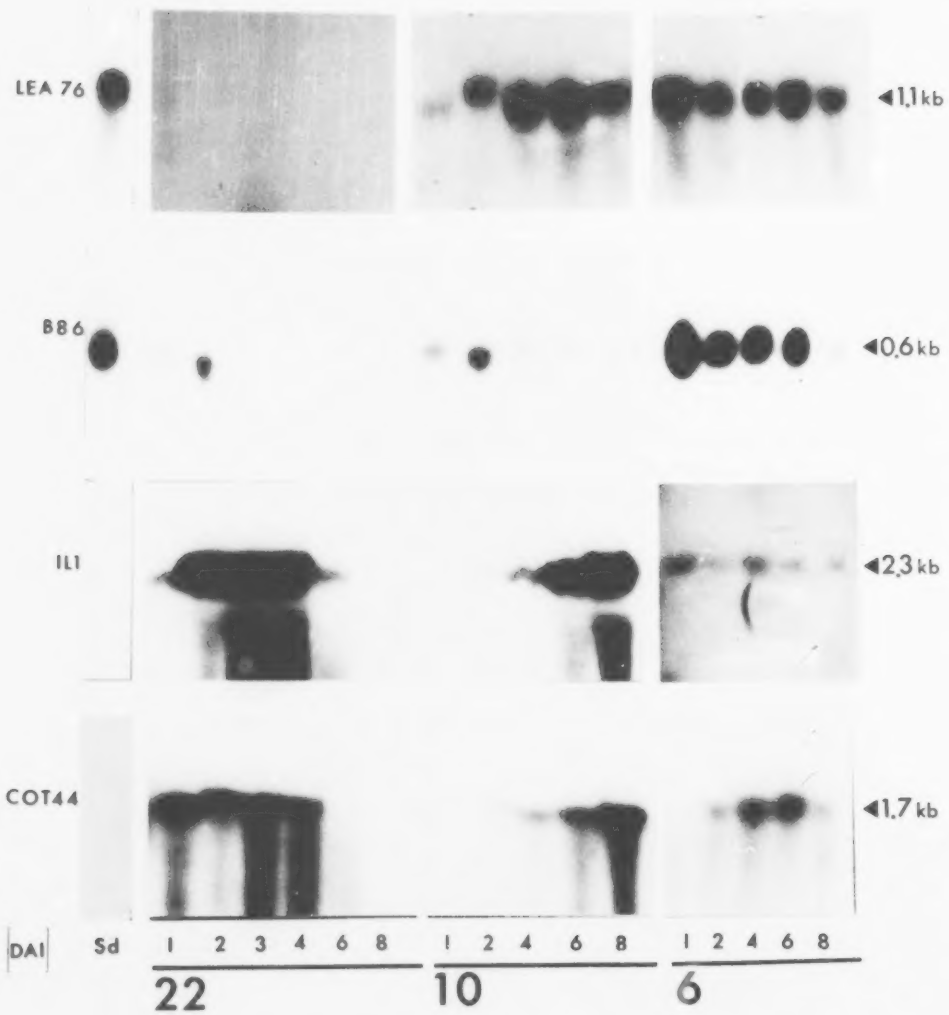
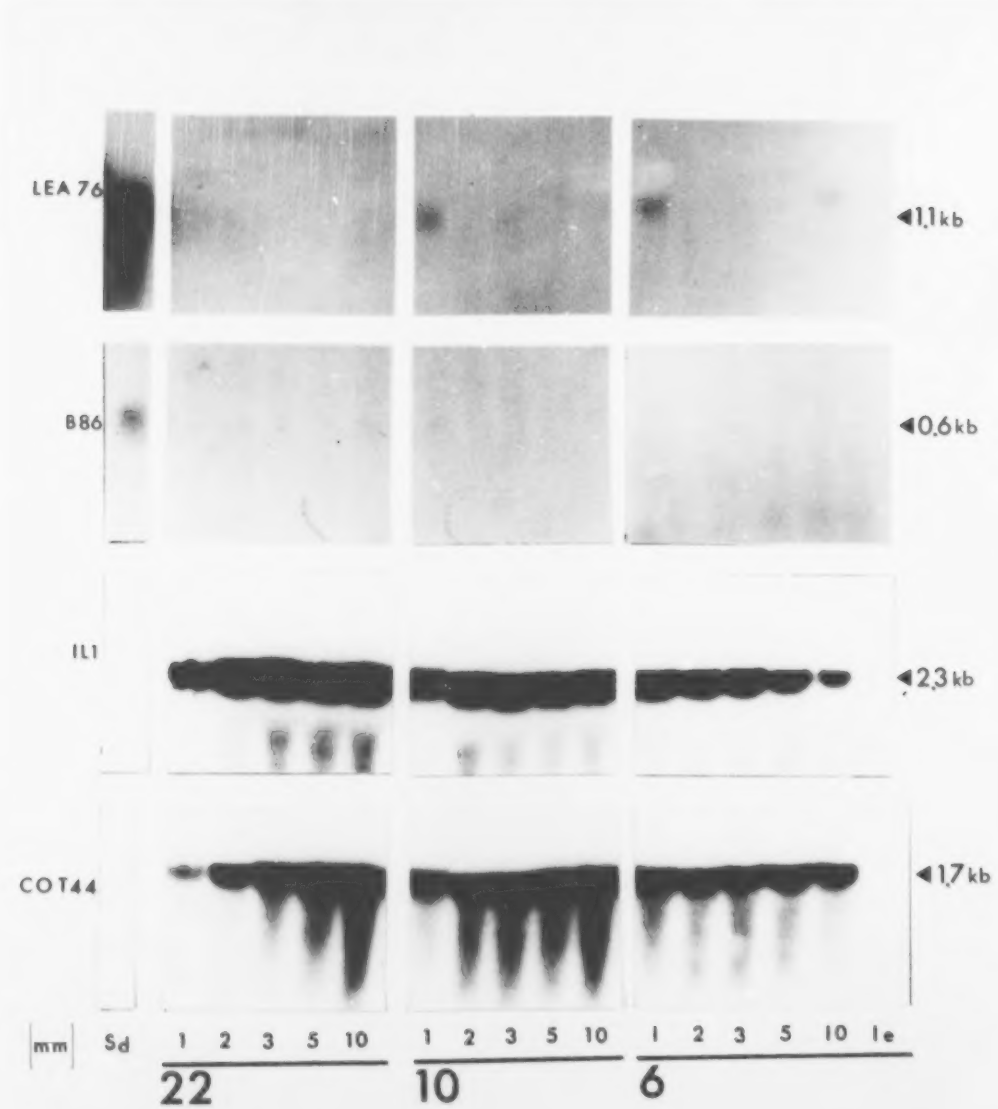

LEA 76
◀1,1 kb
B86
◀0,6 kb
IL1
◀2,3 kb
COT44
◀1,7 kb
DAI
Sd
1 2 3 4 6 8
1 2 4 6 8
1 2 4 6 8
22
10
6

Figure 4-3. Northern blot analysis of the developmental molecular markers LEA 76, B86, IL1, and COT 44 during specific stages after germination as measured by radicle length (mm) at 22, 10, and 6°C. Each lane represents the total extracted RNA (20µg) from populations of 20-30 seedlings collected from each temperature condition from *B. napus* cv. Westar seed. RNA extraction, blotting, and hybridization conditions are outlined in the material and methods section. Sd, denotes total RNA extracted from mature seed; le, denotes total RNA extracted from leaf tissue.


LEA 76
1.1kb
B86
0.6kb
IL1
2.3kb
COT44
1.7kb
mm
Sd
1 2 3 5 10
1 2 3 5 10
1 2 3 5 10 le
22
10
6

conditions (Fig. 4-3).

3.3. Isoctirate Lyase (ICL) Transcripts. Over the course of imbibition at 22°C, ICL transcripts were expressed to high levels on 2 DAI, remained high until 4 DAI, and decreased thereafter to low levels on 6 DAI (Fig. 4-2). In comparison, high levels of expression were delayed until day 6 at 10°C. At 6°C, only low levels of transcript were detected under the same hybridizing and autoradiogram developing conditions.

Expression at 22 and 10°C was similar at the same developmental stages, with a rapid increase during growth to 1 mm radicle length, followed by a gradual increase thereafter (Fig. 4-3). At 6°C, transcript level increases corresponding with 1 mm radicle length decreasing gradually thereafter.

3.4. COT 44 transcripts. To follow the onset of early seedling growth a cDNA recognizing COT 44 messages was employed. At 22°C, COT 44 was not detected in the dry seed but increased dramatically within 1 DAI, remained high until 4 DAI, and then decreased to low levels by 6 DAI (Fig. 4-2). At 10°C, we observe a gradual increase in COT 44, with high levels on 8 DAI. In comparison, the 6°C sample displayed low levels of COT 44 throughout the experimental time period.

At both 22 and 10°C, COT 44 increased gradually from moderate levels at 1 mm radicle to high levels at 10 mm radicle length (Fig. 4-3). At 6°C, the COT 44 was present in moderate levels at 1 mm radicle and decreased gradually thereafter.

3.5. Western Analysis. Quantitative changes in ICL protein were followed under each temperature regime (Fig. 4-4). Under optimal temperature conditions we observed a large increase, peaking at 3-4 DAI, followed by an immediate decline in ICL to undetectable levels by day 10. In the 10°C sample, ICL increased to moderate levels by day 4, and decreased slightly until day 10. At 6°C ICL varied little over the 10 days.

3.6. ABA Analysis. At 22°C, ABA levels declined in an almost linear fashion over 6 days (Fig. 4-5). At 10°C, ABA levels decreased over the

Figure 4-4. Western blot analysis of isocitrate lyase (ICL) from total protein (10 µg/lane) isolated 1-10 days after imbibition (DAI), during germination and early seedling growth, under the optimal and suboptimal temperature conditions in *B. napus* cv. Westar. The temperatures tested were 22, 10, and 6°C, with (◂) denoting the 63 kD polypeptide.

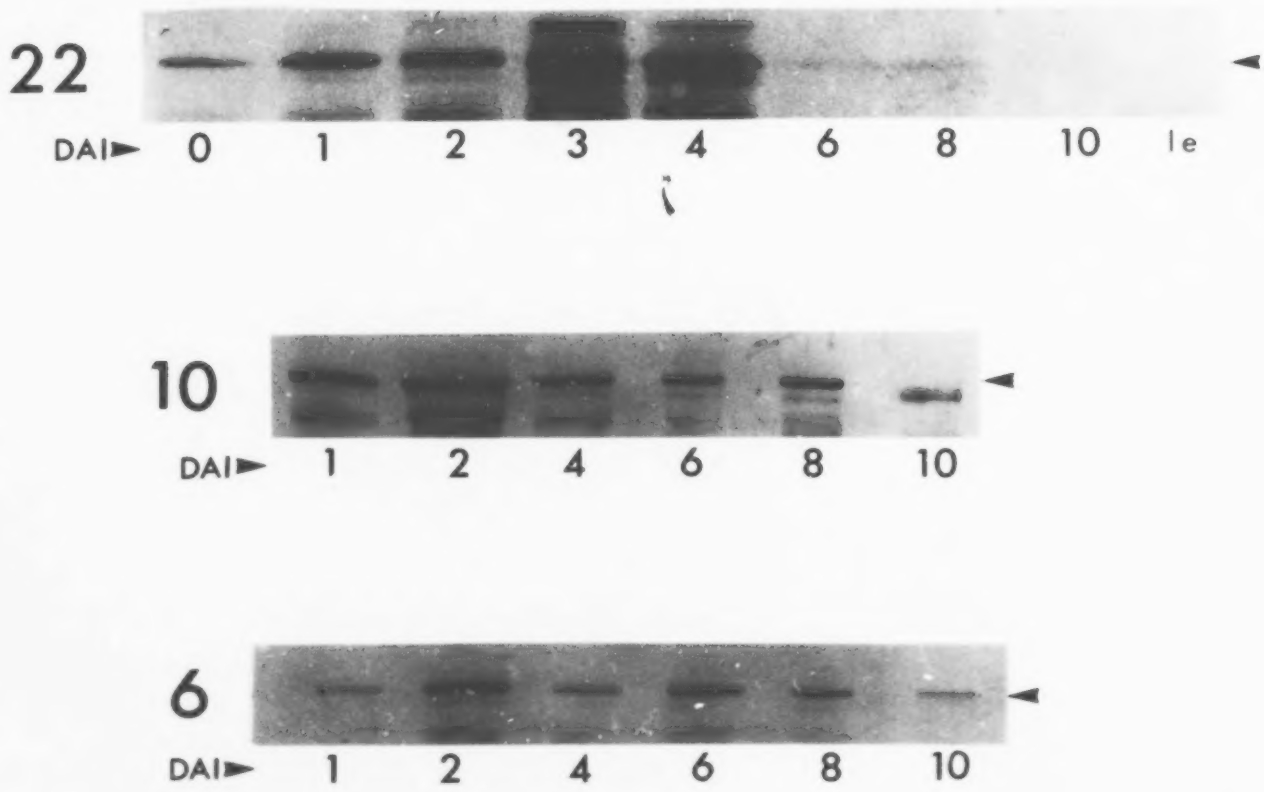
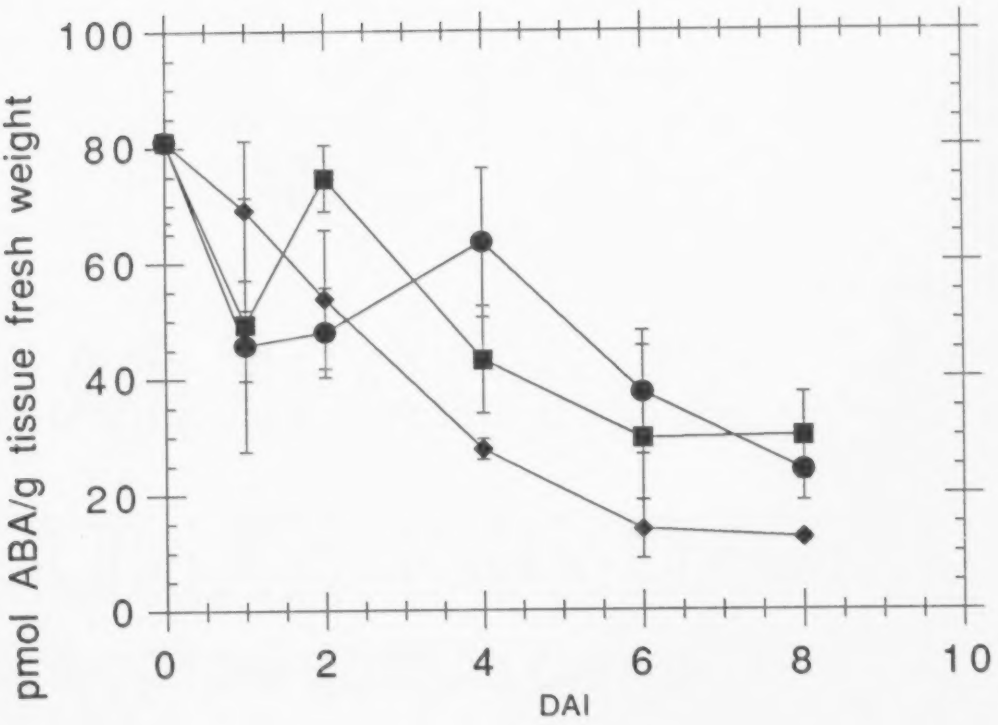

22
DAI
0
1
2
3
4
6
8
10
le
10
DAI
1
2
4
6
8
10
6
DAI
1
2
4
6
8
10

Figure 4-5. Endogenous ABA levels measured at each temperature condition ((♦) 22°C, (■) 10°C, (●) 6°C) over the course of imbibition (DAI) in *B. napus* cv. Westar. The samples were measured on the basis of fresh weight for the reasons outlined in materials and methods. Each value represents the mean of three independent replicates ± 1 SD.

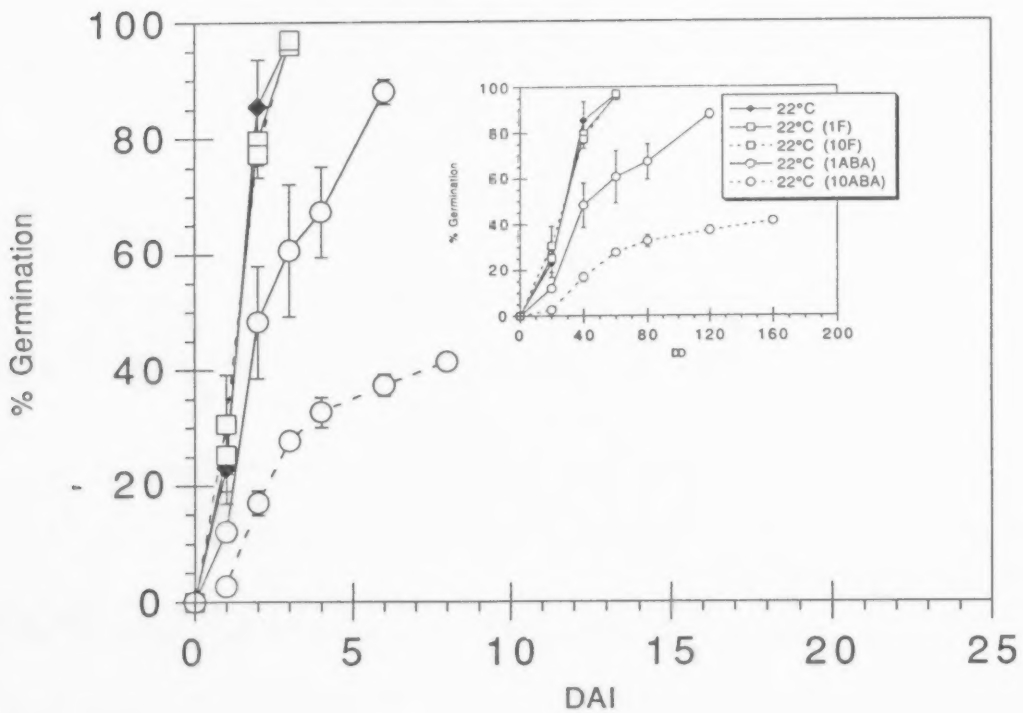


first DAI, increased again on 2 DAI and then declined thereafter. A similar scenario occurred at 6°C, with an initial decline in ABA levels (2DAI) followed by an increase at 4 DAI with levels decreasing thereafter.

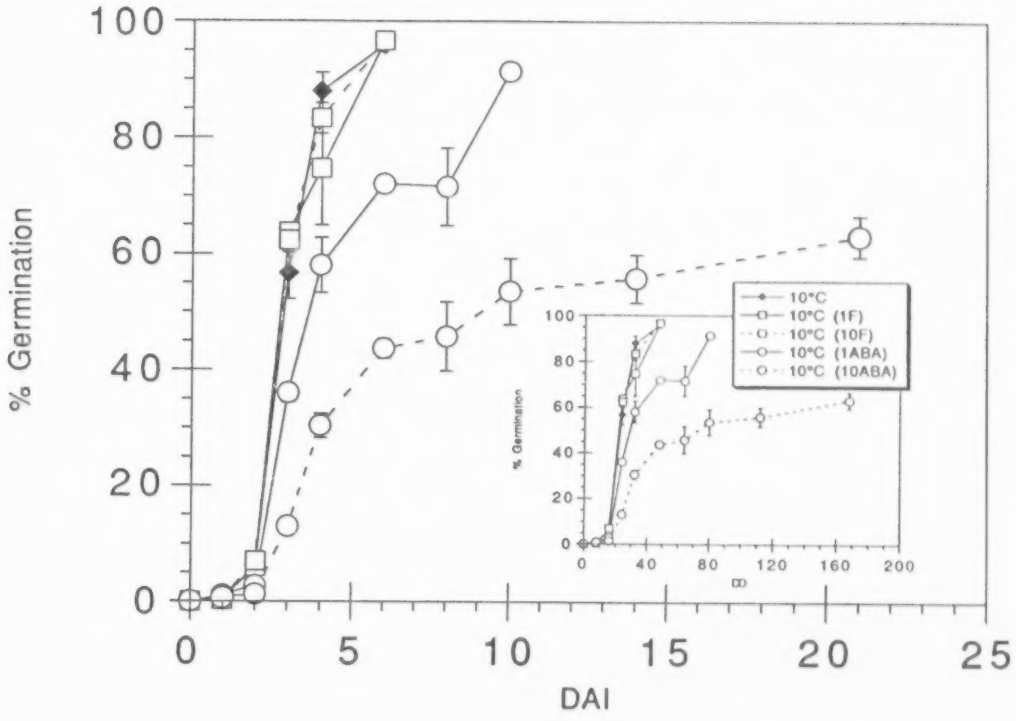
3.7. ABA and fluridone effects during low temperature germination. The interaction between seed germination and ABA content was examined further by treating seed with ABA or fluridone (Fig. 4-6 A,B,and C). ABA concentrations of 1 and 10 µM decreased germination rates at all 3 temperatures; 22, 10, and 6°C, with 10 µM being more inhibitory. Fluridone concentrations of 1 and 10 µM stimulated germination at 6°C , but had little effect at the higher temepratures.

Figure 4-6. Germination potential of *B. napus* cv. Westar seed in the presence of 1 or 10 µM ABA, or in the presence of 1 or 10 µM fluridone in comparison to seed imbibed in only ddH_2O. Seed germinated according to the procedures outlined in materials and methods at A. 22°C, B. 10°C, and C. 6°C. Each value represents the mean of three independent replicates ± 1 SD.

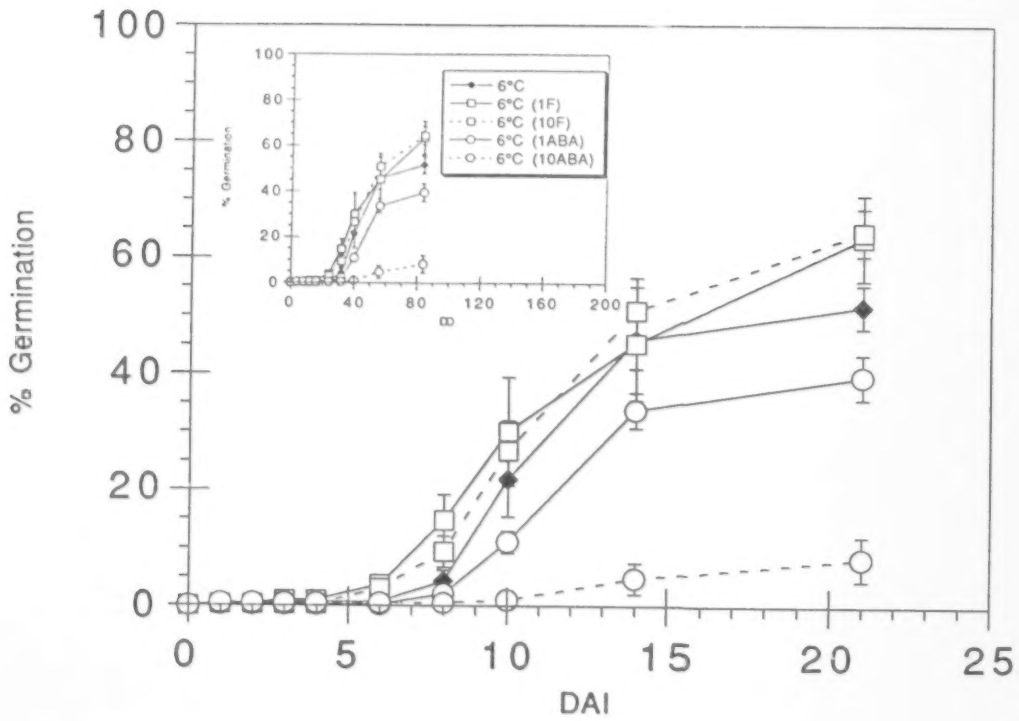
A.



B.


100
80
60
40
20
0
% Germination
0
5
10
15
20
25
DAI
10°C
10°C (1F)
10°C (10F)
10°C (1ABA)
10°C (10ABA)
% Germination
0
40
80
120
160
200

c.



4.0. Discussion.

Again, seed imbibed in the optimal condition (22°C) germinated rapidly, reaching 95% by day 3. Seed imbibed at 10°C required 8 days to attain the same level of germination. At temperatures below 10°C, germination was low. At 6°C, germination only reached 10% by day 8, and there was practically no germination at 2°C. These observations have been previously made in several related studies (Acharya *et al.*, 1983; Kondra *et al.*, 1983; King *et al.*, 1986; Barber *et al.*, 1991; Wilson *et al.*, 1992). The germination potential of the seed was tested again to ensure that cv. Westar seed (Lendholm) retained the same germination potential as seed used in the initial studies (Chapter 2 and 3) and revealed that the viability of the seed was still the same.

Previous observations may indicate an inability of the seed to switch developmental programs under low temperature conditions. Therefore, we employed molecular markers associated with specific developmental programs. These markers were chosen to sequentially delineate the changes from embryogenesis to germination to early seedling growth.

In *B. napus*, LEA 76 increases dramatically in developing seed (26-35 DAF), remains at high levels in the dry seed (60 DAF), and declines rapidly upon imbibition during germination (24 HAI) (Harada *et al.*, 1989). Another *Lea* message, B86, declines rapidly after 48 hours (Raynal *et al.*, 1989). These changes are strictly correlated with germination. As such, results from the present study indicate that the switch from embryogenesis to germination is delayed. Both LEA 76 and B86 declined rapidy after imbibition at 22°C. However, at 10 and 6°C we observed an initial decrease followed by an increase. Thereafter, the messages began to decrease again. These observations have been obtained in repeated studies using the same material and probes, and therefore these results are reliable and

valid as they relate to the present study. These observations suggest that the steady state levels of these *Lea* messages actually increased during the course of low temperature imbibition. This did not simply reflect lower germination rates in the seed samples, as low levels of LEA76 were detected in 1 mm radicle seedlings at 10 and 6°C, but not at 22°C.

The peaks in LEA transcripts were preceded by transient increases in ABA. In previous studies, ABA has been demonstrated to induce LEA expression (Harada *et al.*, 1989; Galau *et al.*, 1991; Jakobsen *et al.*, 1994). This can be correlated with the attainment of desiccation tolerance. Therefore, the presence of LEA transcripts and delay in germination at the low temperature may reflect an inability of the seed to switch completely from the embryogenesis to germination programmes.

Results from Northern and Western blots of ICL suggest that 10°C causes a temporal delay in development, while at 6°C there is poor co-ordination of lipid mobilization. ICL transcripts are present during late embryogeny and in the mature seed (Comai *et al.*, 1989). Upon imbibition the transcripts increase rapidly, remain high over the first six days of imbibition, and decline thereafter to undetectable levels (Comai *et al.*, 1989; Ettinger and Harada, 1990; Comai *et al.*, 1992). At 6°C, it was apparent that the seedling must rely heavily on preformed ICL to degrade lipids. Transcript levels were low and decreased as the radicle elongated to 10 mm. This may be why germination and seedling growth is poor at 6°C. In contrast, at 22 and 10°C, ICL transcript levels were high in seedlings having a 10 mm radicle.

Comparisons between Northern and Western data at 22 and 10°C suggest that low temperature alters the relative rate of post-transcriptional processes. At 22°C, ICL protein and message levels are correlated, whereas at 10°C, transcript levels are proportionately higher. This may suggest

decreased translation or increased turnover of ICL at the lower temperatures. In *B. napus* the expression and accumulation of both ICL and MS have been demonstrated to be under transcriptional control, but post-transcriptional processes lead to quantitative differences (Comai *et al.*, 1989). Therefore, post-transcriptional processes may be impaired or delayed at temperatures below the optimum.

Results from the seedling specific marker, COT 44, were consistent with the idea that low temperature leads to a temporal delay at 10°C and poor development at 6°C. COT 44 is present in low amounts during late embryogeny, increases during germination (24 HAI), and remains high during postgermination in the cotyledons (Harada *et al.*, 1988; Dietrich *et al.*, 1989). The same temporal pattern of expression was observed in seeds imbibed at 22°C with levels peaking on 3 DAI, concurrent with 95% germination. At 10°C, high levels of COT 44 were delayed until 8 DAI, but were still concurrent with 95% germination. Decreasing levels of COT 44 during seedling growth at 6°C were indicative of developmental impairment.

The delays observed in the developmental switch from embryogenesis to germination may involve ABA. The results from the present study show that low temperature germination is associated with an initial decrease followed by a subsequent increase in endogenous ABA levels. The experiment employed could not distinguish whether the secondary peak arose from a re-induction of ABA synthesis or from conjugated storage forms of ABA present in the dry seed (Salisbury and Ross, 1985), as the ELISA procedure employed will only detect free *cis, trans*(+)ABA (Martens *et al.*, 1983).

Exogenous ABA concentrations as little as 10 µM (Schopfer and Plachy, 1984) will inhibit the germination of *B. napus* seeds, and concentrations ten fold higher will maintain seeds in a quiescent state for long periods of time (Schopfer and Plachy, 1985). Singh and Sawhney

(1992) observed a delay and reduction in germination rates between a mutant (*ogura*) and normal line of *B. napus* seed in which the mutant seed contained 4-fold more endogenous ABA.

Results from the present study may indicate that the threshold for ABA to inhibit germination is in the range of 65 to 81 pmol/g fresh wt. Experiments using exogenously applied ABA (1 μM) resulted in delays in germination at 25°C, in seeds possessing 65.2±8 pmol ABA/g tissue (Singh and Sawney, 1992). In the present study dry seed contained 81.03±14 pmol/g tissue ABA. The peaks in ABA fall within this range as well. These peaks were associated with increases in LEA transcript levels and delayed germination.

To further examine the role of ABA in germination, we imbibed seed in the presence of ABA or fluridone. Exogenous ABA reduced the rate and success of germination (Fig. 4-6). At lower temperatures this effect was amplified. (Fig. 4-6, B and C). If ABA were inhibiting germination at low temperature, then fluridone would be expected to overcome the inhibition. Fluridone inhibits carotenoid biosynthesis by blocking the conversion of phytoene to phytofluene by inhibiting phytoene desaturases, thereby inhibiting the accumulation of ABA (Gamble and Mullet, 1986). The results clearly show that fluridone increases the percent germination at 6°C.

It may be possible that exposure of the seed to low temperature could lead to higher endogenous ABA levels over the course of imbibition resulting in a delay in germination. The effects of temperature on endogenous ABA levels have been well documented (Zeevaart and Creelman, 1988; Hallgren and Oquist, 1990). For example in potato, low temperatures led to transient increases in the endogenous levels of ABA (Chen *et al.*, 1983).

In conclusion, we observed delays in the switch from embryogenesis to germination and from germination to early seedling growth based on the

results obtained with the molecular markers for ICL and COT44 transcripts. The delays in these messages appear to be the result of thermodynamic constraints at lower temperatures. The reduction in seedling growth at 6°C is probably a result of both thermodynamic constraints and developmental impairments. In addition, ABA appears to play a role in the temporal delays observed.

5.0. References.

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Chapter 5. The Enhancement of Low Temperature Germination in *Brassica napus* cv. Westar: Genotype Versus Phenotype.

1.0. Introduction.

The Westar cultivar was developed by Agriculture Canada in 1982 (Canola Growers Manual, 1991). Cultivars are developed for a distinct set of characteristics, and in the case of *B. napus* must meet the standards set out by the Canadian General Standards Board for canola. Each cultivar possesses characteristics distinct from other cultivars of the same species, and these characteristics must breed true from generation to generation (Fehr, 1987). This being the case for Westar, the genotypic variability within the Westar genotype is relatively small. Indeed, PCR analysis within the near isogenic Westar genotype reveals around 6% variation in the alleles (Dr. A. Laroche, personal communication).

Genotype is defined as the heritable or genetic composition of an individual plant (Stoskopf, 1981). Phenotype is the physical appearance of the plant resulting from the action of the environment on the genotype (Stoskopf, 1981). In *B. napus*, when environmental conditions are controlled for and maintained between seedlots of the same cultivar, the resulting germination displayed at low temperature is a measure of each population's genotype. Differences within a population under different environmental conditions, e.g. varying low temperature germination, is then a measure of the effects of the environment on the genotype, i.e. a measure of the phenotype.

To date, the attempt to increase low temperature emergence in *B. napus* has met with little success. Acharya *et al.* (1983) determined that for the cultivar Westar, 10°C was the optimal temperature to select for superior low temperature germination. However, attempts to select for rapid germination at 10°C produced inconsistent results (Acharya *et al.*, 1983). A follow up study also determined that selections within Westar did not lead to better germination performance at low temperatures (King *et al.*, 1986). Similarily, attempts to correlate seed characteristics,

such as seed size with germination potential have also met with little success. Studies relating seed size to low temperature germination revealed that seed size could not be correlated with successful germination for either *B. napus* or *B. campestris* (Barber *et al.*, 1991).

The deposition of storage reserves during embryogenesis has been well studied and characterized (Crouch and Sussex, 1981; DeLisle and Crouch, 1989; Murphy *et al.*, 1989; Hoglund *et al.*, 1992; Cummins *et al.*, 1993; Tzen *et al.*, 1993). The subsequent utilization of the storage reserves after germination is essential to the success of early seedling growth. Despite this, no study has related the deposition of storage reserves to the subsequent success of germination. We observed previously (Chapter 2) that H had more protein than L on a per seed basis, but that L had greater germination at 10 and 6°C. These results suggest a need to clarify the relationship between protein and germination potential.

From the previous studies we demonstrated that differences exist between Westar seedlots with respect to low temperature germination and early seedling growth (Chapter 2). In addition, we demonstrated the complex coordination of storage reserve mobilization is important to the success of early seedling growth after germination is completed (Chapter 2 and 3). The delays and reductions observed in germination and early seedling growth are also related to developmental cues which may be related to hormonal control (Chapter 4). Bearing these factors in mind we wished to assess the effects of environment and genotype on the success of low temperature germination.

2.0. Materials and Methods.

2.1. Plant material. The *B. napus* cv. Westar seed used in the present study was previously designated Lendholm and Holmstrom (L and H) (Chapter 2).

2.2. Plant growth and selfing conditions. S_0 seed was produced from both parental seedlots (L and H) by selfing the plants. Plants were grown in the greenhouse with a 12 hr photoperiod (350 $\mu E/m^2/s$), at 25°C, and watered daily until maturity (approximately 90 days). Seed was placed at a depth of approximately 1 cm, in 6 inch pots, in a matrix containing 0.42 peat:0.42 vermiculite: 0.16 sand (v:v:v), 135 g dolomite lime, 120 g Osmocote (18-6-12), 70 g superphosphate, 2 g fritted trace elements, and 1 g iron chelate sequesterene. After bolting, plants were bagged with plastic that allowed for gas exchange and prevented any outcrossing. As *B. napus* is self-fertile (Fehr, 1987), self-pollination occurred and seeds were allowed to grow until maturity (approximately 99 days).

2.3. Seed germination. Seeds were germinated in the manner previously described in Chapter 2 for all tests. Germination potential was measured according to Wilson *et al.* (1992), and data were collected from each DAI at each temperature condition. The fresh weights of mature parental (L and H) and selfed seed (LS_0 and HS_0) were measured.

2.4. SDS-PAGE and Western blot analysis. Protein extraction, quantification and electrophoresis conditions were followed in the same manner described in Chapter 3. Protein transfer and blotting conditions were also followed according to the methods described previously (Chapter 3). After transfer of total protein (1 µg/lane for oleosin or 10 µg/lane for cruciferin) onto nitrocellulose (Schleicher and Schuell, Keene, New Hampshire), blots were analyzed for either cruciferin or oleosin in the same manner described in Chapter 3.

2.5. Bagged seed. During the growth of Westar plants new flowers were

hand pollinated and tagged for identification. *B. napus* flowers are receptive to fertilization 3 days prior to and 3 days after flowering, in addition, the process from pollination to fertilization takes 12-18 hours (Dr. G. Stringham, personal communication). Flowers were emasculated just prior to anthesis. The gynocecium were pollinated by hand. This involved rubbing pollen from receptive flowers directly on stigmas of the emasculated flowers. The siliques were then bagged at the appropriate time with paper bags wrapped with aluminum foil to prevent light from reaching the siliques. The bags were fastened loosely to the peduncle to allow for gas exchange. The siliques were bagged on 0, 12, and 28 DPA. Concurrently, additional flowers were hand pollinated and tagged for identification, but not covered. Once the uncovered siliques had reached 60 DPA, and, therefore, the seed had reached maturity, the seed from the bagged and unbagged conditions was collected and tested for germination at 22°C.

Additional samples were collected and tested for dry weight. Dry weight was determined by drying seed populations of 100 seed in a vacuum oven overnight.

3.0. Results.

3.1. Germination potential of S_0 seed. At 22°C there was no difference in germination between the parental and S_0 seed in either the L or H seedlot. Both seedlots in the parental and S_0 populations reached 95% germination within 4 days. However, at lower temperatures differences in germination occur. In the L seedlot, germination at 10°C was slightly better in the S_0 population requiring only 6 days to attain a high level of germination (Fig. 5-1). In the H seedlot germination at 10°C was much more rapid in the S_0 seed than in the parental seed, requiring 8 days compared to 12 days to reach high levels of germination (Fig. 5-2). At 6°C, we observe large increases in germination in the L and HS_0 seed over that of the parental seed (Figs. 5-1 and 5-2). There was no improvement at 2°C.

3.2. Seed weight. H parental seed displayed the largest seed weight, followed by the L S_0, L, and finally the H S_0 seed (Table 5-1). There was no consistent difference between the generations.

3.3. Western blots. Western analyses of L and H (parental and S_0) seed for oleosin and cruciferin were performed. L displayed higher cruciferin (on a total protein basis) over that of the H seed when the parental seed was compared (Fig. 5-3A). This was associated with higher germination in the L seedlot in comparison to the H seedlot at 10 and 6°C. There was slightly higher cruciferin in the LS_0 population in comparison to the HS_0 seed, and this was associated with similar differences in germination between the selved seedlots at 10 and 6°C. Between the L parental and LS_0 populations, slightly higher amounts of cruciferin were present in LS_0. In relation to the germination potential LS_0 displayed slightly better germination at 10°C and higher germination at 6 C. Between H and HS_0, cruciferin was present in much higher contents in the HS_0 population than

Figure 5-1. Differences in germination potential between the parental and S_1 seed of the Lendholm seedlot. The seed sources tested were L; (■) 10°C, (●) 6°C, and (▲) 2°C, LS_0; (□) 10°C, (○) 6°C, and (△) 2°C. Each value represents the mean of three independent replicates ± 1 SD.

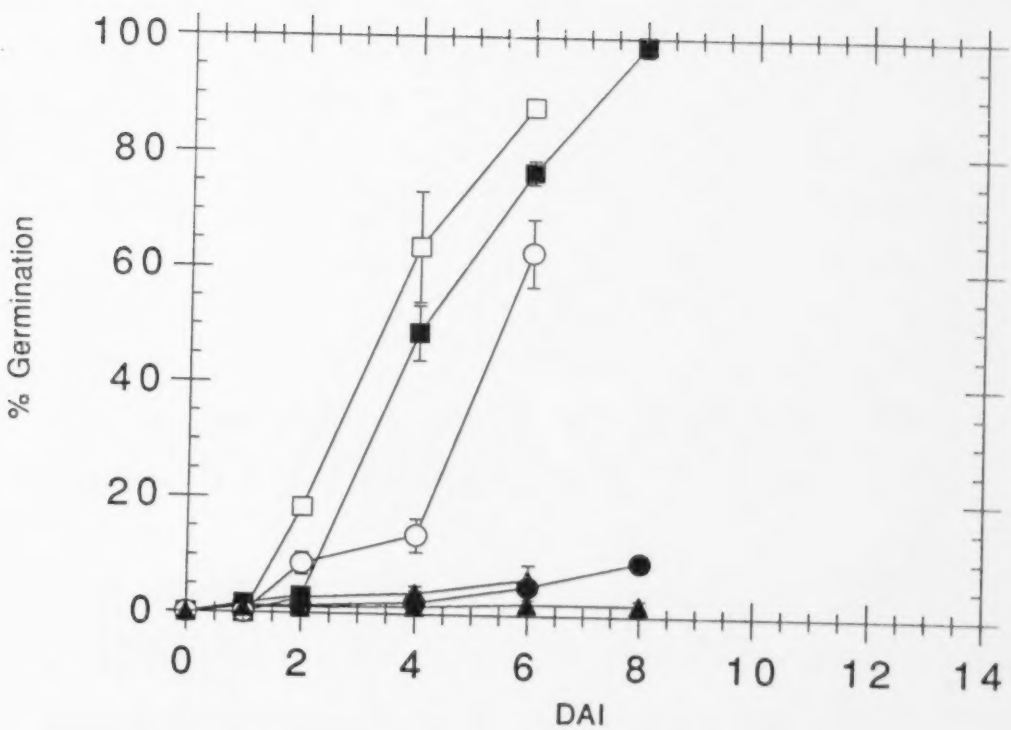


Figure 5-2. Differences in germination potential between the parental and S_0 seed of the Holmstrom seedlot. The seed sources tested were H; (■) 10°C, (●) 6°C, and (▲) 2°C, HS_0; (□) 10°C, (○) 6°C, and (△) 2°C. Each value represents the mean of three independent replicates ± 1 SD.

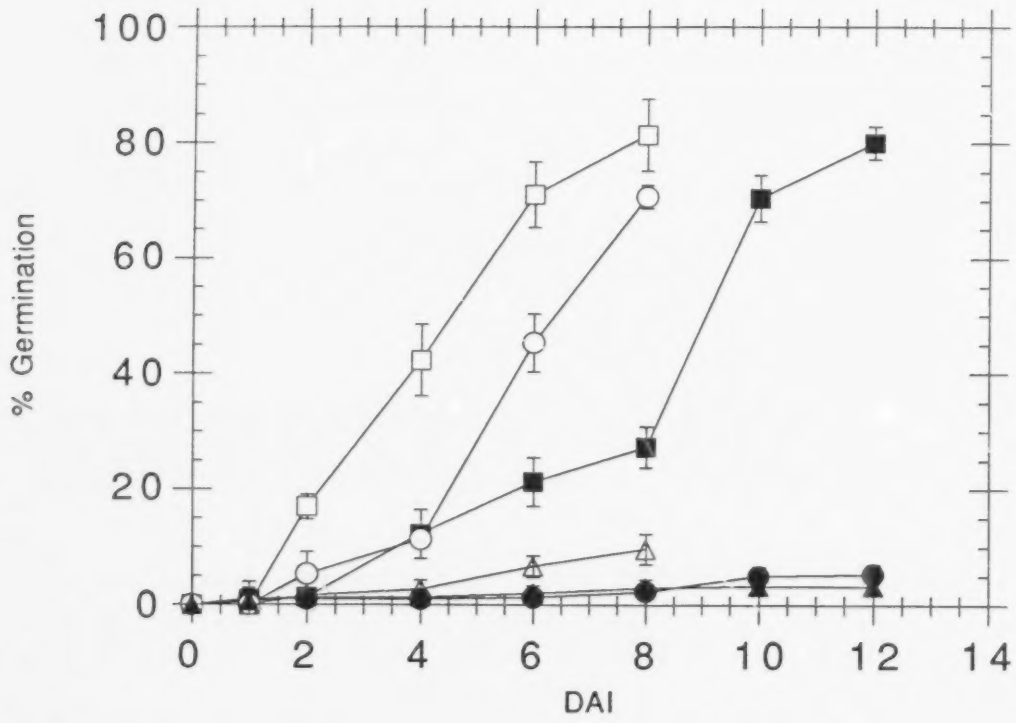
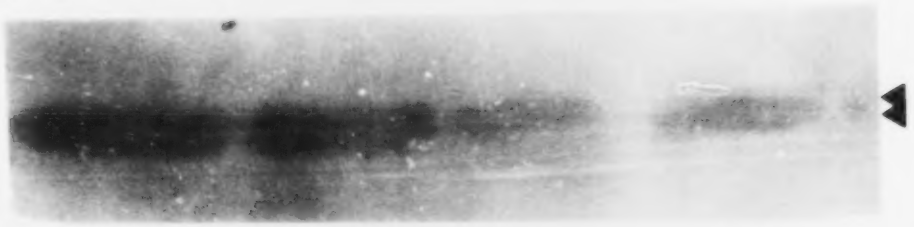


Table 5-1. Fresh weight values obtained for the parental L and H populations and the selfed seed from each of these populations (LS_o and Hs_o). Also included in the table are percent damaged seed and moisture content.

Seed Population	Fresh weight/100 seed (mg/100 seed)	% damaged seed	% moisture
L	310±3	2.3±0.4	1.6±0.3
H	384±4	2.0±0	7.9±0.3
LS_o	364±14	6.7±1.9	14.6±2.4
HS_o	287±1	4.0±0.1	11.2±0.9

Figure 5-3. Western blot analysis of storage proteins between the parental L and H populations and their corresponding S_0 populations. A. High molecular weight of cruciferin α-subunits detected in protein blots containing 10 µg/lane total protein. B. 20 kD oleosin subunit detected in protein blots containing 1 µg/lane total protein.

A



L LS_o H HS_o

B

L LS_o H HS_o

displayed in H. Germination was much higher at 10 and 6°C in the HS_0 population in comparison to the H population. Therefore, increases in germination at 10 and 6°C correlated with cruciferin content in the parental and selfed populations. There were no consistent differences in oleosin content, nor were there consistent differences between the generations (Fig. 5-3B).

3.4. Bagged seed. As expected, the dry weight of bagged seed was low, with dry weight increasing from bagging at 0DPA to unbagged seed (Table 5-2).

Germination tests were performed at 22°C on bagged and unbagged seed (Fig. 5-4). Both control seed (unbagged) and 28DPA bagged seed germinated well, reaching 95% within 3 days. The 23 DPA seed reached a maximum of 30% germination at 4 DAI, and the 18 DPA seed only reached 3% germination.

Protein profiles for bagged and unbagged seed loaded on a per embryo basis revealed that 0 and 12 DPA bagged seed had low protein content in addition to containing less cruciferin and napin (Fig 5-5). There was little difference between 28 DPA bagged seed and the unbagged control seed.

Further insight into the effect of bagging on protein accumulation was gained by running Western blots of oleosin and cruciferin. The amount of oleosin present in the seed was stable through to maturity (on the basis of 1 µg total protein) (Fig. 5-6a). Cruciferin, however, increased throughout the study period. The levels of cruciferin (10 µg total protein) were found to be lower in the bagged conditions in comparison to the unbagged control condition (Fig. 5-6b).

Table 5-2. Dry weight values for unbagged and bagged seed (0, 12, and 28 DPA) of *B. napus* cv. Westar. The dry weight measured was on the basis of 100 mature seed from the control (unbagged condition) and the bagged seed. Bagged seed was collected at the same time as the unbagged seed after approximately 60 DPA. The conditions below (0, 12, and 28 DPA) correspond to the bagging dates as outlined in materials and methods.

Seed	**Dry weight/100 seed (mg/100 seed)**
Sd (unbagged)	503.6±8.7
28 DPA	376.3±19.2
12 DPA	199.5±24.6
0 DPA	101.7±8.7

Figure 5-4. Germination potential of unbagged and bagged seed at 22°C. (♦) unbagged, (□) 28DPA bagged, (○) 12 DPA bagged, and (△) 0 DPA bagged. Each value represents the mean of three independent replicates ± 1 SD.

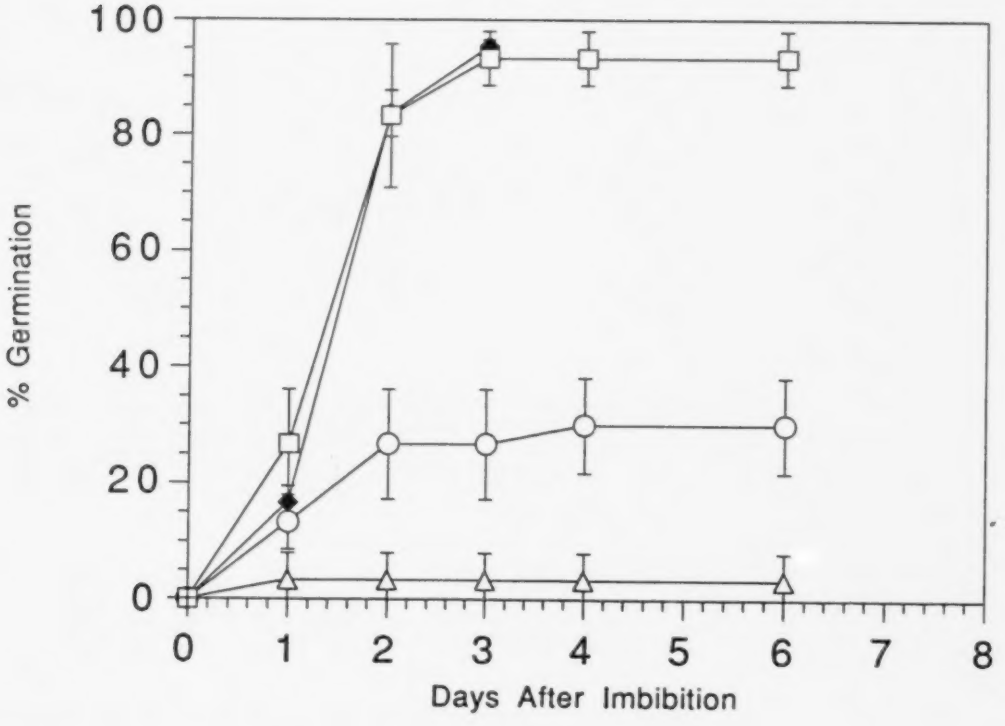


Figure 5-5. Protein profiles of protein extracted from unbagged and bagged (0, 12, and 28 DPA) seed. Samples were separated on 15% SDS-PAGE and were loaded on a per seed basis. Sd denotes mature dry seed from the unbagged condition, 28 DPA denotes mature seed from siliques bagged 28 days after hand pollination, 12 DPA denotes mature seed from siliques bagged 12 days after hand pollination, and 0 DPA denotes mature dry seed collected from siliques bagged directly after hand pollination. M indicates molecular weight markers.

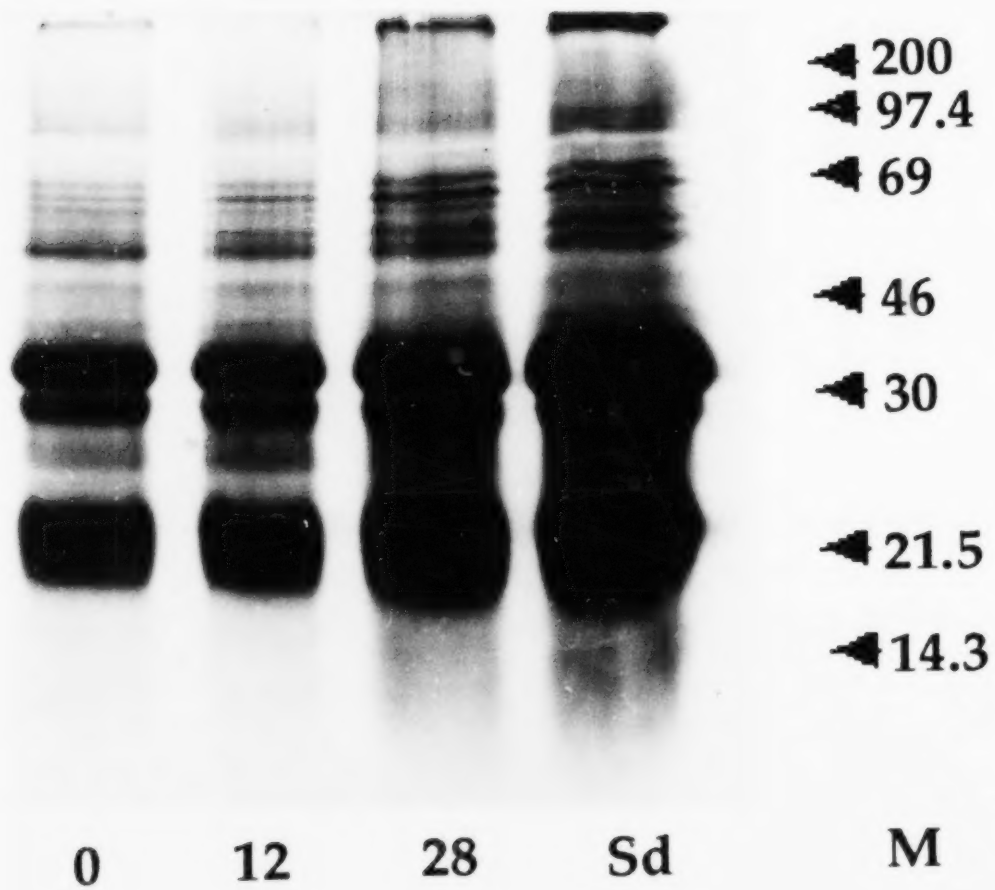

200
97.4
69
46
30
21.5
14.3
0
12
28
Sd
M

Figure 5-6. Western blot analysis of unbagged and bagged (0, 12, 28 DPA) seed on the basis of total protein. A. Employing antibodies directed against the 20 kD oleosin subunit (1 µg/lane total protein, and B. employing antibodies against the α-subunits of cruciferin (10 µg/lane total protein). Arrows denotes the subunit(s) detected with each antibody. O, 12, and 28 DPA indicates the date of bagging with seed collection at maturity, and Sd indicates seed collected from the same plants from unbagged siliques (i.e. unbagged seed) collected at maturity.

A

Sd O Sd 12 Sd 28

B

Sd O Sd 12 Sd 28

4.0. Discussion.

Differences between *B. napus* cultivars are relatively small. On the basis of 198 cDNAs used to map polymorphisms between two cultivars, Westar and Topas, approximately 92 (47%) resulted in differences (Landry *et al.*, 1991). When only seedling specific cDNAs were mapped, only 6% (8 loci) variability between the cultivars was detected (Landry *et al.*, 1991). As both L and H seedlots were certified cv. Westar (Allard, 1960), the seed would be nearly genetically identical (Fehr, 1987). However, commercial seedlots of *B. napus* are heterogeneous and maintain some residual heterozygosity (Landry *et al.*, 1991). Indeed at the optimal temperature of 22°C we observed no difference in the success or rate of germination between the L and H seedlots. The differences became evident at lower temperatures (Fig 5-1 and 5-2). At 10°C, we observe slight differences in the rate but not the success of germination over the experimental time frame. These differences become increasingly more evident at 6°C. This was true for both the parental and S_0 populations, suggesting slight genotypic variations exist within the Westar cultivar.

Although seed size could be expected to correlate with germination and early seedling growth rates, our results demonstrated a lack of correlation, with H having larger seeds than L, LS_0 being larger than L, and HS_0 being smaller than H. Similarily, Barber *et al.* (1991) determined that seed size is not a selection marker for seed vigor at low temperature within the Westar cultivar.

To our knowledge there has been no attempt to correlate the success of low temperature germination to the presence of storage protein present in the mature dry seed. Cruciferin accounts for 50-60% (Crouch and Sussex, 1981; Hoglund *et al.*, 1992) and oleosin accounts for 10% (Huang, 1992; Cummins *et al.*, 1993) of the total protein in mature *B. napus* seed, and both are mobilized after germination (Finkelstein and Crouch, 1984; Murphy *et al.*, 1989; Hoglund *et al.*, 1992; Huang, 1992).

Comparisons between germination rates and cruciferin content suggest

a good correlation between protein content and germination. In general genotypic variation was expressed in storage protein accumulation, with L and LS_0 being superior to H and HS_0. This again suggests a genotypic advantage in L.

The effect of environment could also be assessed between the seedlots. LS_0 and HS_0 seeds developed under optimal conditions in the greenhouse, therefore, phenotypic differences in germination potential at low temperature and storage protein deposition could be assessed. Both S_0 populations displayed higher germination rates, and higher germination success at 6°C. This indicates that environmental conditions during embryogenesis play a major role in the seeds' ability to germinate at low temperatures. Certainly, environmental factors during seed development and maturation have been shown to alter the success of germination (Gray and Thomas, 1982; Taylor *et al.*, 1991). These environmental factors (drought, light, minerals, etc) influence the maternal plant and alter seed filling by stressing the whole plant. Our S_0 populations were grown under controlled conditions, each plant receiving the same nutrients, water, and light. Therefore, superior low temperature germination of the S_0 population resulted from an optimal environment for seed development. Another factor which must be considered is the storage time of the mature seed. Both the LSo and HSo seed was tested for germination potential immediately after maturation. The L and H parental seed was tested for germination potential when the seedlots were received from seed growers, and therefore was approximately 6 months to 1 year old. The effects of storage on the seeds viablity has been documented with respect to storage lipids (Vertucci, 1992). It is possible that the longer storage times impacted on the seeds ability to germinate at low temperature by depleting some of the stored lipids. During Vertucci's (1992) study the amounts of the amounts of storage proteins were not assessed. In the present study the amounts of oleosin did not correlate with germination potential in the parental or selfed populations, and therefore is not believed to play a

factor in the present study.

In order to further examine the effect of environment on seed germination, we reduced the amount of light received by the silique and seed. As expected, the treatment reduced seed size (Table 5-2) and storage protein deposition significantly (Fig. 5-6), and as a consequence reduced the success of germination (Fig. 5-4). Although the bagged and unbagged seed displayed little or no difference in the amount of oleosin accumulated from 12 DPA on, there were differences in cruciferin deposition. These differences between the proteins reflect the known differences in the timing of their accumulation and expression (Crouch and Sussex, 1981; Hoglund *et al.*, 1992; Huang, 1992; Wilen, 1992; Cummins *et al.*, 1993 Tzen *et ak.*, 1993). Of greater interest was the fact that bagging led to a reduction in the quantity of cruciferin. This may suggest that light is required for the proper accumualtion, i.e. correct processing and deposition, of cruciferin.

In conclusion, results from this study indicate there is a small genetic component present in Westar seedlots that enables the seed to produce higher levels of storage reserve proteins and to germinate more rapidly, leading to a higher percentage of germination. There is also a large environmental component. The effects of genotype and environment are not necessarily noticeable under optimum conditions. These results taken together implore producers to improve seed management practices and explore new avenues of research in order to enhance the ability of canola or produce new lines conferring superior low temperature germination. One environmental component that has received little attention during seed maturation, but plays a major role in the overall amounts of storage protein deposited during seed maturation is light. Canola is a chlorophyllous seed during embryogenesis, and, therefore, light may be playing a pivotal role during cell expansion and storage reserve deposition. Study of this aspect requires a different experimental approach, as there is no way to uncouple the effects of low light on the

silique from the effects on the seed.

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Chapter 6. The Accumulation of Storage Proteins in Microspore Derived Embryos of *Brassica napus* cv. Topas.

1.0. Introduction.

Embryogenesis provides a unique window of development to study morphological, physiological, biochemical and molecular events. During zygotic embryogenesis storage reserves are laid down in preparation for germination and early seedling growth (Thomas, 1993; West and Harada, 1993). In *B. napus* approximately 20% of the dry weight of mature seed constitutes protein (Finlayson, 1976). Cruciferin accounts for approximately 60% of the total protein, and napin accounts for an additional 20% (Crouch and Sussex, 1981; DeLisle and Crouch, 1989; Hoglund *et al.*, 1992).

Cruciferin is a globulin like (Murphy *et al.*, 1989) hexamer with a molecular weight of 300 kD (Rodin and Rask, 1990a). Each subunit is composed of an α-heavy chain (approximately 30 kD) and a β-light chain (approximately 20 kD) (Rodin and Rask, 1990b; Sjodahl *et al.*, 1991). Polypeptides are joined to each other by a disulfide bond (Rodin and Rask, 1990b), and models have been presented postulating its conformation *in vivo* (Plietz *et al.*, 1987). Napin is an albumin like (Lonnerdal and Janson, 1972; Dalgarrondo *et al.*, 1986) dimer with a molecular weight of 14 kD, with one heavy polypeptide (9 kD) disulfide bound to a light polypeptide (4 kD) (DeLisle and Crouch, 1989).

Both storage proteins are processed from precursors (DeLisle and Crouch, 1989; Rodin and Rask, 1990b; Breen and Crouch, 1992; Hoglund *et al.*, 1992), and the mature holoprotein is deposited in dense protein bodies via the Golgi apparatus as the seed matures (Chrispeels, 1991; Hoglund *et al.*, 1992). Although gene expression, protein accumulation, and product deposition have been extensively studied, the processing of cruciferin and napin from their precursor form into the mature holoproteins found in protein bodies of mature seeds has still not been fully demonstrated (Murphy *et al.*, 1989; Hoglund *et al.*, 1992).

In *B. napus* cv Topas, tissue culture techniques have been developed to produce large quantities of embryos (Telmer *et al.*, 1992, and

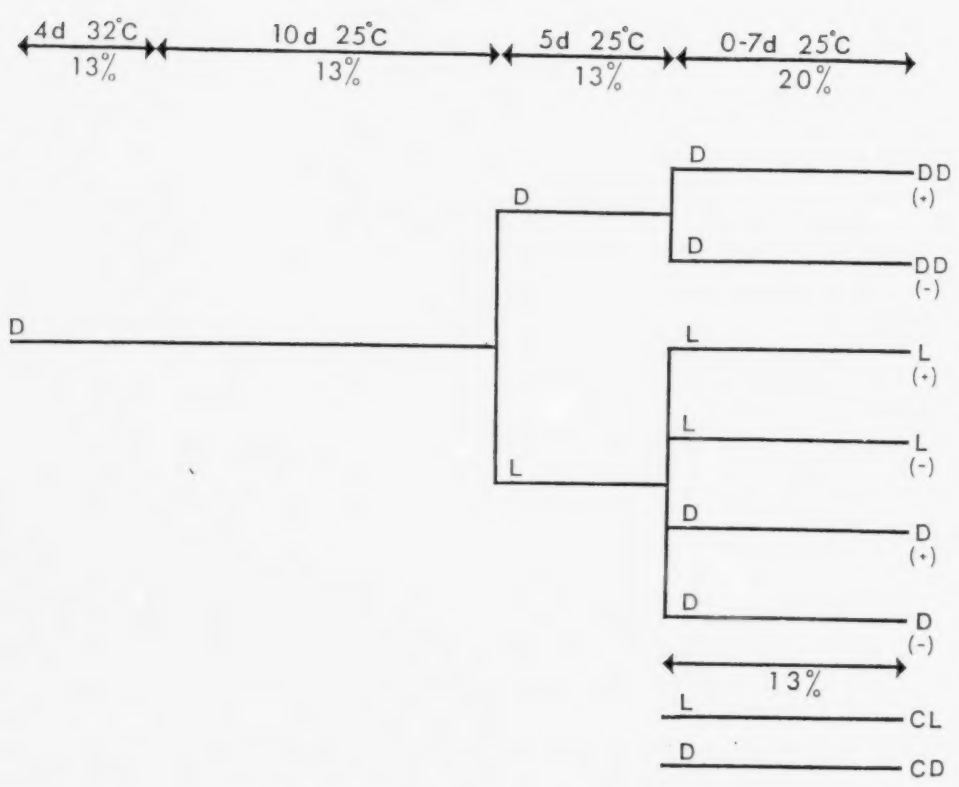
references therein). These advances have enabled researchers to compare MDE to the zygotic embryos in biochemical and molecular terms. MDEs mimic the zygotic system in relation to lipid biosynthesis and accumulation (Taylor *et al.*, 1990; Chen and Beversdorf, 1991; Pomeroy *et al.*, 1991; Taylor *et al.*, 1992). It has further been shown that oilbody-associated proteins (oleosins) accumulate similarly to those in zygotic embryos (Wilen, 1992). In relation to storage protein gene expression, both cruciferin and napin are similar to those in zygotic embryos, however, the MDEs do not accumulate the end products (Finkelstein and Crouch, 1986; Wilen *et al.*, 1990; 1991; Wilen, 1992). However, Kennedy (1993) in a recent study observed the accumulation of polypeptide bands corresponding to the molecular weights of cruciferin and napin under certain culturing conditions in the light. This system could be employed first to determine whether the bands are indeed cruciferin and napin, and thereafter utilize these conditions to study the accumulation of storage proteins. MDEs are an ideal system to apply external factors, as they are free from the surrounding sporophytic tissue and maternal influences are removed. Thus they provide an opportunity to study the effect of reduced light on storage protein gene expression (Finkelstein and Crouch, 1896; Wilen *et al.*, 1990; 1991). In addition, we wished to gain a better understanding of the regulation of storage protein biosynthesis in MDEs.

2.0. Materials and methods.

2.1. Plant material and growth conditions. The plant material used was *B. napus* cv. Topas T4079. Culvtivar Topas is used because of the ease of inducing embryogenesis in isolated microspores. To date the optimal conditions to induce MDEs in cv. Westar have not been elucidated. The use of a different cultivar would not result in gross differences in storage protein expression because of the relatively small difference in genotype between Westar and Topas. Therefore, the observations obtained in the present study can be confidently related to what would be occurring in Westar. Plants were grown and cultivated according to the methods previously mentioned in Chapter 5. After plants had started bolting they were removed from the greenhouse and transferred to phytotrons. Growth in the phytotrons took place at 4°C with a photoperiod of 12 hr light/12 hr dark (350 $\mu E/m^2/s$).

2.2. Microspore production. Microspore isolation and haploid embryo development were carried out according to Kennedy (1993). After microspore isolation in modified Lichter medium (Lichter, 1982; Orr *et al.*, 1990) containing 13% sucrose (NLN-13%), and embryogenesis induction at 32°C, microspores were incubated at 25°C in the dark with mild agitation (shown in Figure 6-1). The microspores were then transferred aseptically to fresh 13% sucrose (NLN-13%). MDEs were screened through sterile polyester mesh with a 530 µM pore size (PeCap, Tetko Inc., Elmsford, N.Y.) to ensure isolation of MDEs in the heart to torpedo stage of embryogenesis. Thereafter, MDEs were returned to the dark or placed in the light (80-100 $uEm^{-2}s^{-1}$ from fluorescent and incandescent lights placed approximately 18 inches from plates) for 5 days (Fig. 6-1). After the 5 day pretreatment microspores were again seived through sterile polyester

Figure 6-1. A schematic drawing depicting the conditions in which the microspores were cultured. Embryos were isolated during the 0-7 day region indicated by the upper time line. DD denotes embryos subjected to a 5 day dark pretreatment followed by transfer into 20% NLN and placed in culture media containing ABA (+) or no ABA (-). L and D denote embryos subjected to a 5 day light pretreatment and returned to the light (L) or dark (D) with (+) or without (-) ABA in 20% NLN. CL denotes embryos given the 5 day light pretreatment followed by 7 days in continuous light while remaining in 13% NLN. Likewise, CD denotes embryos given the 5 day dark pretreatment followed by 7 days in continuous dark while remaining in 13% NLN.


4d 32°C
13%
10d 25°C
13%
5d 25°C
13%
0-7d 25°C
20%
D
D
D
DD
(+)
D
DD
(-)
L
L
(+)
L
L
(-)
D
D
(+)
D
D
(-)
13%
L
CL
D
CD

mesh as above to isolate MDEs at the torpedo to mid-cotyledonary stage of embryogenesis and then aseptically transferred to fresh 20% sucrose (NLN-20%) medium and returned to the light (L) or dark (D) with (+) or without (-) 50 µM ABA as denoted in Figure 6-1. Two additional samples were also cultured; one sample was placed in continuous light while remaining in 13% sucrose (denoted as CL), and the other sample remained in continuous dark while remaining in 13% sucrose (denoted as CD). MDEs were collected from each time interval over sterile nylon mesh (Nitex, Tetko Inc., Elmsford, NY) (1 mm pore size) and flash frozen in liquid nitrogen. From this procedure, MDEs examined for napin and cruciferin were developmentally similar to zygotic embryos from the torpedo (day 0) to cotyledonary stage (day 7) of embryogenesis.

2.3. Protein Analysis. MD embryos were ground in 80% acetone (in an ice bath). The insoluble material was collected by centrifugation at 9000 rpm for 10 min in a SS34 rotor. The supernatant was decanted and the pellet resuspended in 80% acetone. The insoluble fraction was collected as above, and the supernatant again removed. The pellet was then washed in 100% acetone, centrifuged as above, and dried with nitrogen gas. The acetone pellet was then ground in warm (65°C) Laemmli (1970) buffer and placed on ice. Samples were centrifuged, the supernatant retrieved, and placed in a sterile microfuge tube. Total protein concentration was determined according to Bradford (1976) using BSA as a standard as described previously (Chapter 3). Seed and leaf tissue was prepared as above. Protein was boiled for 5 min in Laemmli (1970) buffer, and then loaded on 15% SDS-PAGE mini gels. Gels were run at 180 V for 1 hr. Gels were stained in 0.1% Coomassie blue R-250, 40% methanol and 10% acetic acid for 3 hrs, followed by destaining in 40% methanol, 10% acetic acid for 3 hrs.

2.4. Western blot analysis. Total protein was separated on 15% SDS-PAGE gels as above. Gels were electroblotted onto nitrocellulose for 1 hr at 70 volts according to the conditions of Dunn (1986) as previously

described (Chapter 3). Membranes were blocked overnight in 3% fish skin gelatin (in 1XPBS). Membranes were then incubated with antibody directed against cruciferin (obtained from Dr. ML Crouch) or napin (obtained from AGS) for 1 hr at room temperature. Membranes were washed, incubated with Goat Anti-Rabbit IgG (Sigma), and developed according to the methods of White and Green (1987).

2.5. RNA Extraction and Northern Analysis. Total RNA was extracted from MD embryos, seed and leaf tissue according to Nateson *et al.* (1989). Frozen tissue was ground with hot equilibrated phenol (65°C) followed by additional grinding in an equal volume of 0.2 M NaOAC pH 5.2 and 1/6 volume 10% SDS. The homogenate was transferred to Falcon 2269 polyproplene tubes and incubated at 65°C for 5 min, and centrifuged for 5 min at 5000 rpm in a Sorvall SS34 rotor. The aqueous phase was transferred to a clean tube and an equal volume of $CHCl_3$:IAA was added, vortexed, and centrifuged as above. The aqueous phase was transfered and the RNA precipitated in 1/8 volume 8M LiCl and cold 95% ethanol. The RNA was pelleted by centrifugation in SS34 rotor at 10 000 rpm for 20 min. RNA was redissolved in DEPC-treated water and quantified by absorbance at 260 nm. Total RNA (10 µg/lane) was separated on a formaldehyde denaturing gel according to Fourney *et al.* (1988). RNA was transferred onto Zeta-Probe membrane (Sigma) via capillary action. RNA was crosslinked to membrane by UV light (254 nm) for 2 min at 30 cm. Membranes were then probed with cDNA directed against cruciferin (pC1; Simon et al., 1985) and napin (pN2; Crouch et al., 1983). Both cDNAs were labelled with $dCTP^{32}$ (Ammersham) with a random primer labelling kit (Ammersham) as outlined in materials and methods, Chapter 4. The activity of the probes used in the northern analysis were 2.5 x10 x 10^9 CPM/µg DNA for pC1 and 5.9 x 10 x 10^9 CPM/µg DNA for pN2.

2.6. Hybridization and stripping conditions. Prehybridization and hybridization conditions were followed according to the methods previously

outlined in Chapter 4. Membranes were stripped and reprobed according to the methods also in Chapter 4.

2.7. Storage protein staining in fixed tissue. MDEs were cultured and isolated on various days as outlined above. These MDEs were then fixed in LR white plastic (London Resin Co. Ltd., Basingstoke, Hampshire) according to the following procedure. MDEs were fixed overnight in 2% glutaraldehyde in 50 mM Na_2HPO_4 pH 7.4 at room temperature. The following day, MDEs were rinsed three times for 15 min intervals in 50 mM Na_2HPO_4, pH 7.4. The MDEs were then dehydrated in an ethanol series for 30 min each in 20%, 40%, 60%, 80%, and 95% ethanol. MDEs were then rinsed for 1 hour in LR (London Resin Co.) white plastic:95% ethanol (1:1). This was followed by soaking the MDEs in LR white (100%) overnight. The following day the MDEs were rinsed twice for 1 hour each in fresh 100% LR (London Resin Co.) white plastic. MDEs were then placed in gelatin caps with fresh LR (London Resin Co.) white plastic (100%) and polymerized overnight at 60°C.

Gelatin caps were removed by soaking in warm (65°C) ddH_2O, and were sectioned at a thickness of 1 µm, floated on sterile ddH_2O and placed on a hot plate (65°C) to fix sections to clean microscope slides. The sections were then stained for protein according to the methods of Rahman *et al.* (1992). This involved staining sections for 5 min at room temperature in Buffalo Black NBR (Allied Chemical, Morristown, New Jersey) (0.05% in 0.7% aqueous acetic acid) followed by destaining in 0.7% aqueous acetic acid until background (plastic) was clear.

2.8. MDEs *in vivo* labelling. Microspores were collected on day 3 of the experiment and labelled for 5 hours in the presence of $7.4x10^3$ Bq ^{35}S-labelled methionine (*in vivo* labelling grade, Ammersham). After incubation at the appropriate condition in the presence of radioactive methionine the samples were rinsed with media and returned to culture media in the appropriate condition with 15mg/mL unlabelled methionine (L-methionine, Sigma). The total incorporation of radioactive methionine was

quantified according to the procedures outlined in Chapter 3 after cruciferin was solubilized in extraction buffer: 0.01 M sodium-phosphate buffer, pH 7.5, 0.5 M NaCl, 0.001 M PMSF. Cruciferin was then purified as outline below.

2.9. Cruciferin purification. Cruciferin was purified from labelled microspores according to the methods of Crouch and Sussex (1981). MDEs (about 500-700 mg) were crushed in a mortar on ice with a pestle in 3 mL of extraction buffer: 0.01M sodium-phosphate buffer, pH 7.5, 0.5 M NaCl, and 0.001M PMSF. The homogenate was centrifuged at 15 000 g for 20 min, and the supernatant (with avoiding the lipid pad as much as possible; lipid pad) was filtered through glasswool, and the clear extract was saved and stored at -80°C in 2 ml aliquots. Cruciferin was purified from the crude extract by molecular sieving column chromatography on a BioGel A 0.5 agarose (Bio-Rad Laboratories) column. Samples of 300 000 DPMs were separated on a 1x30 cm column with a flow rate of 45ml/h (Harvard Aparatus Peristalic Pump)with 0.01 M sodium phosphate buffer, pH 7.5, 0.5 M NaCl elution buffer. Prior to the labelling experiment solubilized cruciferin from cv. Topas and Westar seeds were purified as above to determine which fractions contained native cruciferin. Fractions containing native cruciferin were collected (Gilson 203 Fraction Collector) and measured at 280 nm on a Cary 219 (Varian associates, Palo Alto) spectrophotometer for protein concentration. Fractions containing the cruciferin protein were then analyzed for purity on SDS-denaturing PAGE according to the same methods outlined above. Fractions containing cruciferin were isolated from labelled MDEs in the same fashion and run on 15% SDS-PAGE to produce fluorographs of the cruciferin turnover in the MDEs over the experimental time frame.

3.0. Results.

3.1. Total Protein. The results for the untreated embryos are shown in Figure 6-2. Embryos isolated from 13% sucrose (samples CL and CD) after the 7 day period did not accumulate proteins in the molecular ranges of cruciferin and napin (compare to seed in Fig. 6-4). The results for the dark pretreated embryos are shown in Figure 6-3. In DD(+) embryos, protein bands within the molecular weight ranges of cruciferin were present from day 2 through to day 7 (Fig. 6-3). DD(-) embryos did not display protein accumulation in these same ranges. Both DD(+) and DD(-) embryos exhibited weak banding in the range of napin. MDEs preteated with light are shown in Figure 6-4. Embryos grown in the absence of ABA (L(-) and D(-)) show reduced banding in the regions of molecular weight corresponding to cruciferin and napin. Embryos grown in L(+) exhibited high levels of protein in the regions of the same molecular weight as cruciferin and napin (compare to seed). Embryos grown in D(+) also accumulated protein in the same regions as cruciferin and napin (compare to seed), but not as much as embryos grown in L(+). The same patterns of protein accumulation were observed (data not shown), whether protein samples were loaded on the basis of total protein, or on the basis of embryo number.

3.2. Western Analysis. To confirm that the protein bands observed in the SDS-PAGE analysis were cruciferin and napin, antibodies directed against these proteins were employed. MDEs that were dark pretreated are shown in Figure 6-5. In DD(+) embryos, cruciferin accumulated from undetectable levels at day 0 to low levels at day 2 and remained low through to day 7. DD(-) embryos in comparison displayed no cruciferin accumulation through to day 7. Additionally, in both L(-) and D(-) cruciferin was not detected (data not shown).

For MDEs that received the light pretreatment and remained in the light or were returned to the dark are shown in Figure 6-6. In L(+) MDEs, cruciferin increased from undetectable levels at day 1 to levels similar

Figure 6-2. Acetone precipitated protein isolated from CL and CD MDEs cultured until day 7. The total proteins from 500 µg dry weight were separated on 15% SDS-PAGE and Coomassie stained, as outlined in the materials and methods section. (M) denotes molecular markers; CL and CD are embryos cultured according to the treatments outlined in Fig. 6-1.

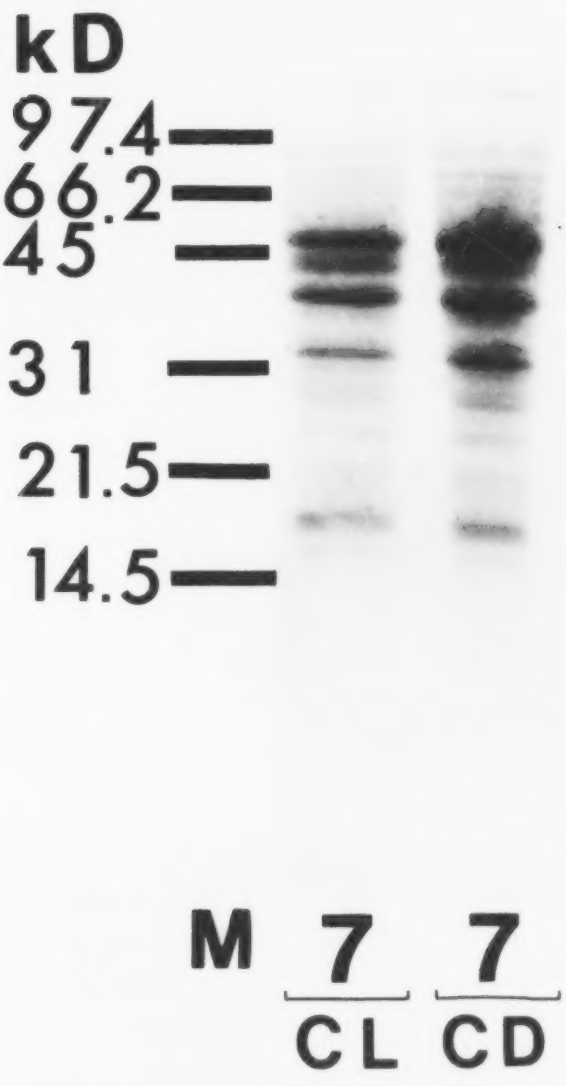

kD
97.4
66.2
45
31
21.5
14.5
M
7
CL
7
CD

Figure 6-3. Analysis of acetone precipitated proteins isolated from MDEs from day 0 to day 7 in embryos from DD(+) and DD(-). The total proteins from 500 µg dry weight were separated on a 15% SDS-PAGE and Coomassie stained. (◂) denotes protein bands in the molecular weight range of cruciferin polypeptides and (▵) denotes bands in the range of napin polypeptides. (M) denotes molecular weight markers; 0-7 denote the time intervals (days) on which MDEs were collected from either the DD(+) or DD(-) culture conditions as outlined in Fig. 6-1.

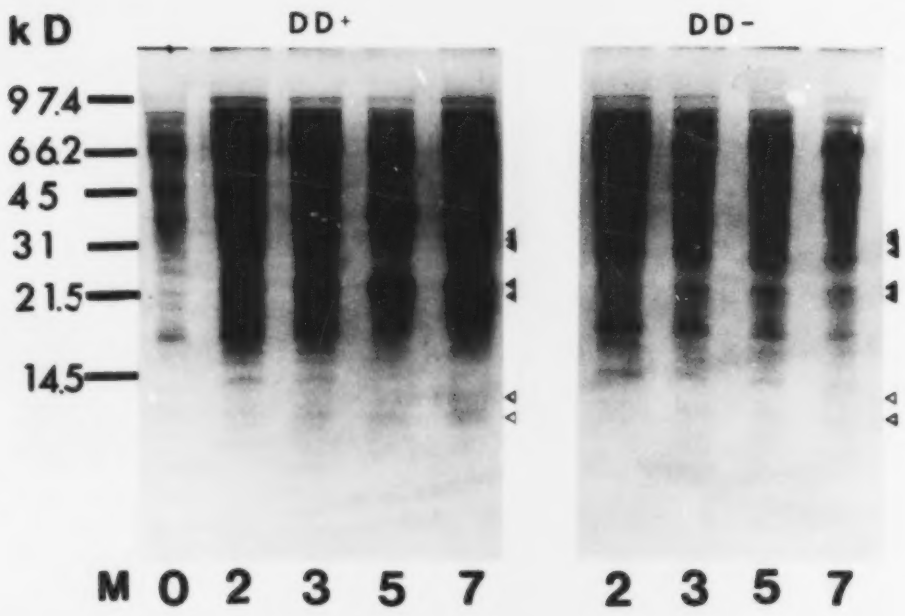

kD
DD+
DD-
97.4
66.2
45
31
21.5
14.5
M 0 2 3 5 7
2 3 5 7

Figure 6-4. Analysis of acetone precipitated proteins isolated from MDEs. The total proteins from 500 µg dry weight were separated on 15% SDS-PAGE and Coomassie stained. Sd denotes protein acetone precipitated from mature dry seed, (◂) denotes protein bands in the molecular weight range of cruciferin polypeptides, and (△) denotes bands in the range of napin polypeptides. Collection days from each of the culture conditions L(+), D(+), L(-), and D(-) are shown. The culture conditions for L(+), D(+), L(-), and D(-) are outlined in Fig. 6-1.

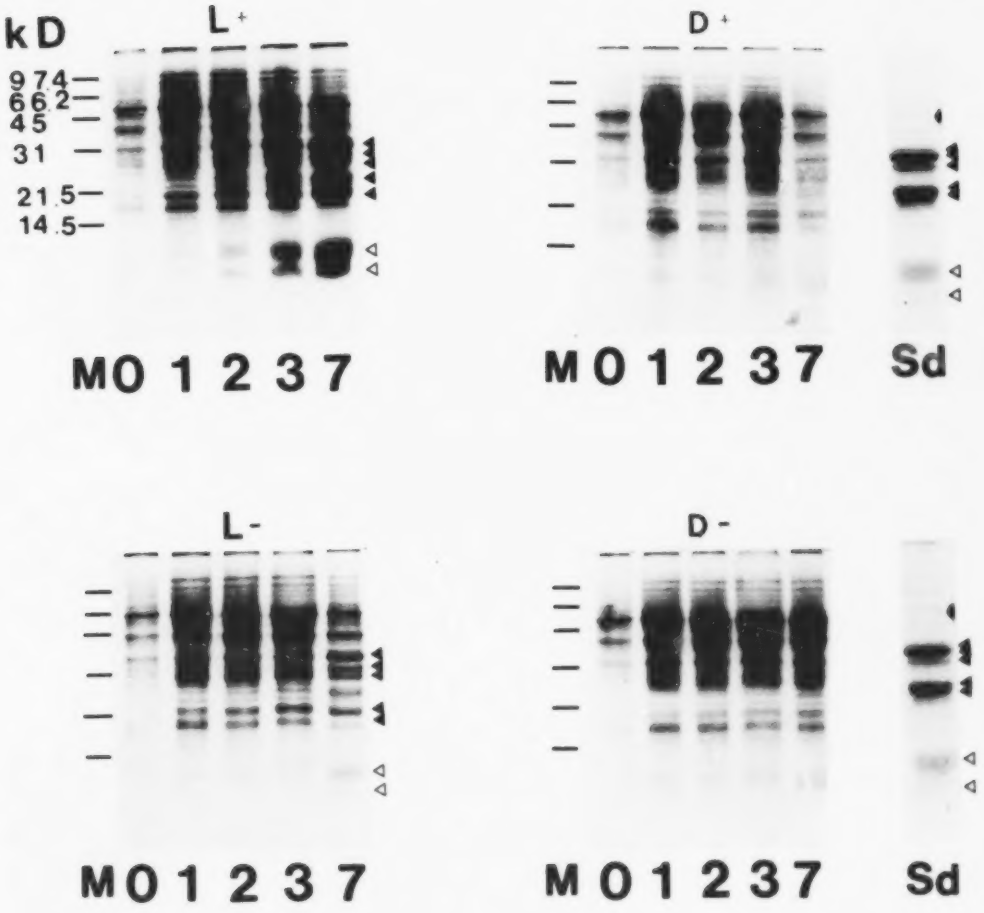

kD
L+
97.4
66.2
45
31
21.5
14.5
M O 1 2 3 7
D+
M O 1 2 3 7
Sd
L-
M O 1 2 3 7
D-
M O 1 2 3 7
Sd

Figure 6-5. Western analysis of cruciferin from total protein (1 µg/lane) isolated from MDEs. The DD(+) and DD(-) MDEs (days 2 to 7) were cultured as outlined in Fig. 6-1. Included is protein isolated from MDEs after the 5 day light preteatment (0(L)) and the 5 day dark pretreatment (0(D)). Arrows (◂) denote the two bands detected by the antibody.

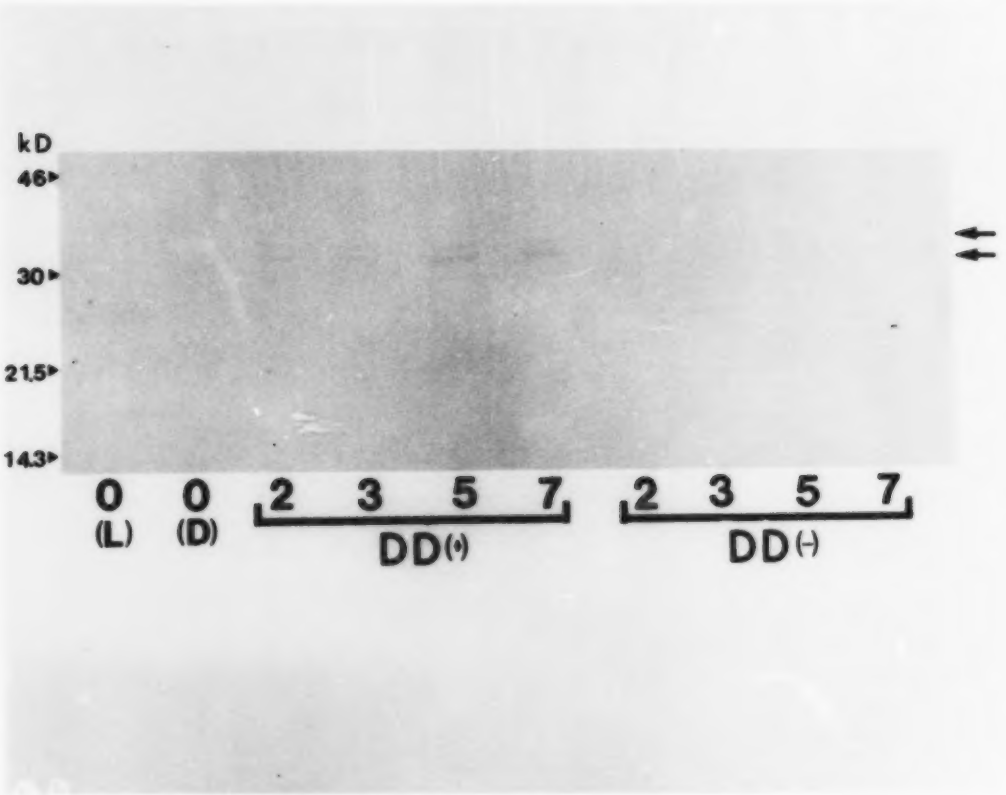
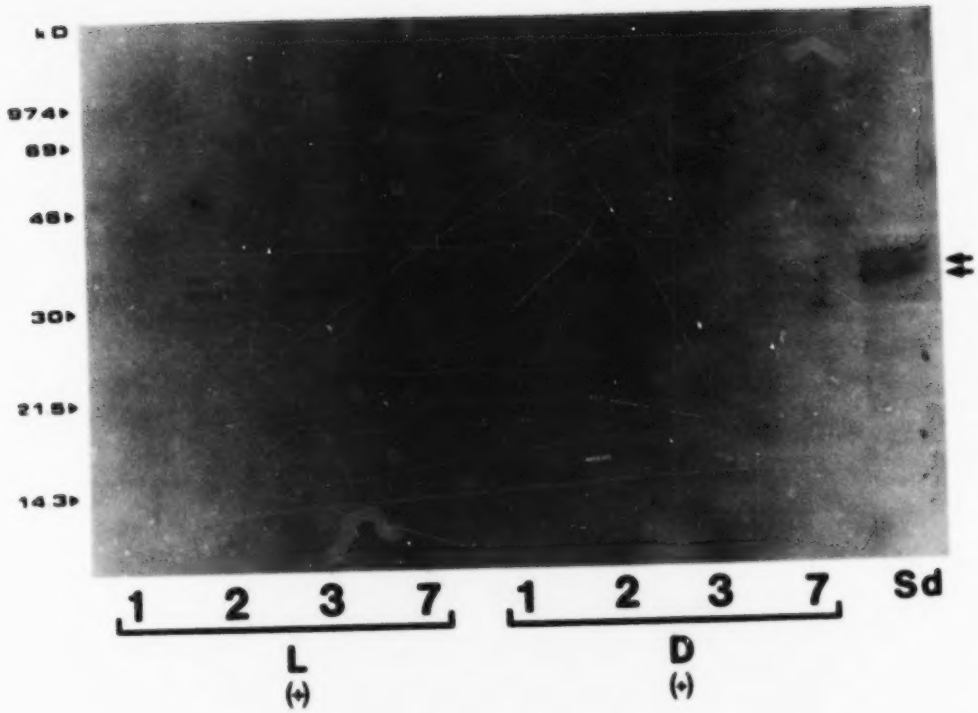

kD
46
30
21.5
14.3
0
(L)
0
(D)
2
3
5
7
DD(+)
2
3
5
7
DD(-)

Figure 6-6. Western analysis of cruciferin from total protein (1 µg/lane) isolated from the L(+) and D(+) MDEs (days 1 to 7). Included is protein isolated from mature dry seed (Sd) for comparison. Arrows (◂) denote the two bands detected by the antibody.


kD
97.4
68
46
30
21.5
14.3
1 2 3 7
1 2 3 7
Sd
L
(+)
D
(-)

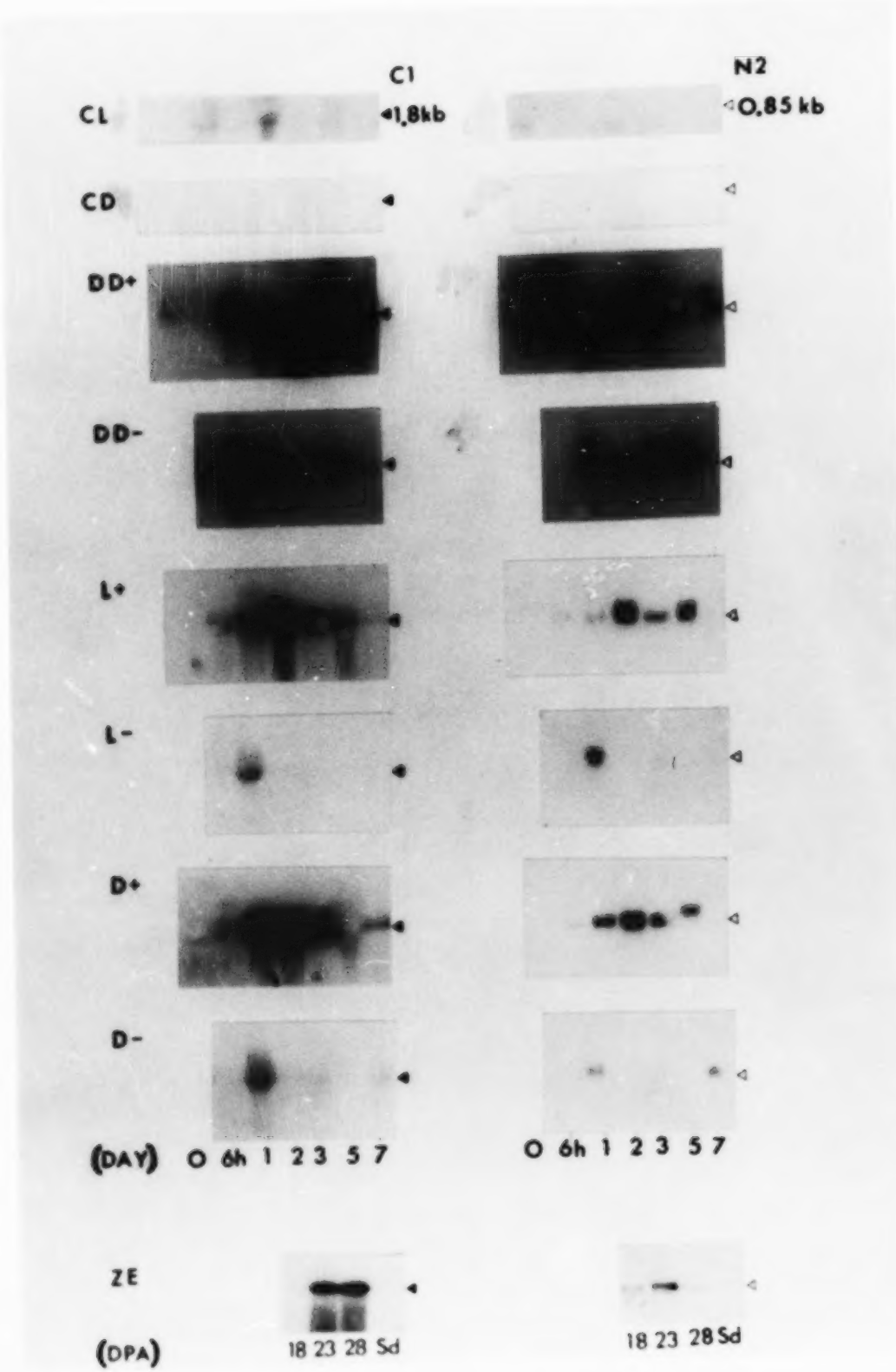
to a mature dry seed by day 7. In comparison, D(+) embryos only displayed low levels of cruciferin at day 7.

The temporal accumulation of napin was also determined (data not shown). Napin was only detected in L(+) embryos at day 7 and in dry seed. Napin was undetectable in all other conditions under the same SDS-PAGE, electroblotting, and immunodetection procedures.

3.3. Northern Analysis. Total RNA was extracted, separated and probed for both cruciferin and napin. In DD(+) embryos, the cruciferin message increased from low levels at day 0 to day 5, after which the message declined slightly (Fig. 6-7). DD(-) embryos also displayed a transient increase in the message from low levels at day 0 to high levels by day 1, declining thereafter to low levels by day 7. Cruciferin levels were transiently high in both L(+) and D(+) (Figure 6-7). In L(+) embryos the cruciferin message increased dramatically from low levels at 6 hr to maximal levels at day 2. After day 2 the message quickly declined to undetectable levels by day 7. In comparison, the D(+) embryos increased steadily in cruciferin from low levels at 6 hr to maximal levels by day 2. After day 2 the message remained high until day 3 after which it quickly declined to low levels on day 7. Under both L(-) and D(-) conditions cruciferin message was low (Fig. 6-7), displaying only moderate levels on day 1. In the control tissues, low levels of cruciferin were detected in the CL condition, but not in the CD condition (Fig. 6-7).

Napin transcript levels were also determined. DD(+) embryos displayed high levels of napin transcripts at day 0 through to day 3, then the transcript began to decline (Fig. 6-7). In comparison, DD(-) embryos accumulated napin transcripts later and to a lesser degree (Fig. 6-7). Under L(+) conditions, the napin transcript increased from undetectable levels at 6 hr to maximal levels by day 2 (Figure 6-7). The message then decreased. In D(+), napin increased from low levels at 6 hr to maximal levels at day 2, declining to moderate levels on day 7. Low levels of napin were detected in the absence of ABA (L(-) and D(-)).

Figure 6-7. Northern analysis of cruciferin and napin transcript levels from total RNA (10 µg/lane) isolated from MDEs (days 0 to 7). Included are cruciferin and napin transcript levels from total RNA (10 µg/lane) isolated during embryogenesis in zygotic embryos (18, 23, and 28 DPA) and mature seed (Sd) for comparison.


C1
N2
CL
1.8kb
0.85 kb
CD
DD+
DD-
L+
L-
D+
D-
(DAY) 0 6h 1 2 3 5 7
0 6h 1 2 3 5 7
ZE
(DPA) 18 23 28 Sd
18 23 28 Sd

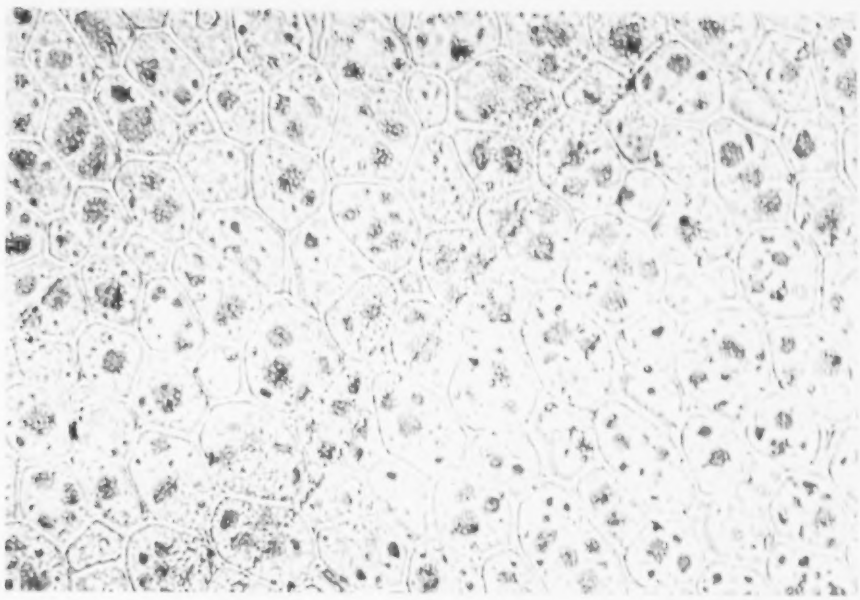
When compared to zygotic tissue, MDEs cultured in the presence of ABA displayed higher steady state levels of both cruciferin and napin (Fig. 6-7). However, in the presence of both ABA and light the transient accumulation and decline of the cruciferin and napin transcripts followed a pattern similar to their zygotic counterparts.

3.4. Protein bodies in MDES. To confirm that cruciferin and napin were sequestered in protein bodies in the MDEs, the tissue was fixed, sectioned and stained for protein. The presence of protein bodies was confirmed only in the culturing conditions in which the presence of cruciferin was detected via western blot analysis (Fig. 6-8). The highest number of protein bodies was detected in the L(+) embryos, followed by the zygotic mature dry seed, DD(+), and D(+) embryos.

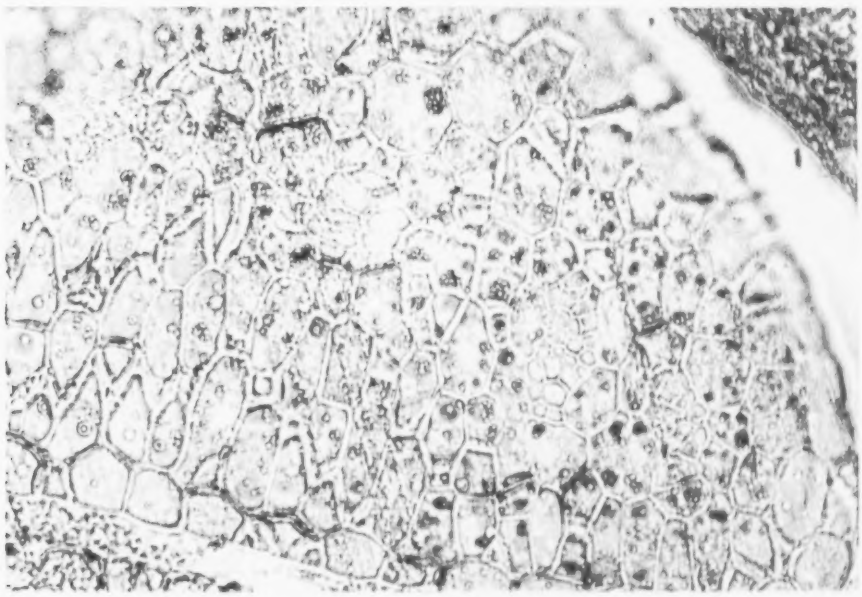
3.5. Cruciferin turnover. Differences in cruciferin and napin accumulation between the different culturing conditions led to the hypothesis that storage protein stability may require or lead to its correct processing and deposition in protein bodies. Polypeptides identified by western blots as cruciferin were observed in the L(+), and DD(+) MDEs (Fig 6-9). A turnover study of cruciferin revealed that the stability of the protein was enhanced in the presence of light, when compared to MDEs cultured in the dark (ABA and osmoticum were present in both conditions) (Fig. 6-9). Whereas cruciferin subunits were rapidly turned over in DD(+), MDEs cultured under L(+) exhibited labelled cruciferin for up to 24 hours. The increase in cruciferin subunits to maximum levels 12 hours after the chase reflects the incorporation of methionine into the mature holoprotein as only native cruciferin was purified. Therefore labelled but unprocessed cruciferin presursor peptides would not appear on the fluorographs and this could reasonably explain the increase observed in the L(+) condition and the low levels of cruciferin in the DD(+) condition.

Figure 6-8. Stained protein bodies (stained blue) from fixed tissue. The procedures for fixing and staining the MDEs and dry mature seed are outlined in materials and methods. Mature seed; W denotes stained sections from cv. Westar, and T denotes stained sections from Topas. Stained sections from CL embryos collected on day 0 and day 7. Stained sections from CD embryos collected on day 0 and day 7. Stained sections from L(+), DD(+), and D(+) embryos collected on day 7.

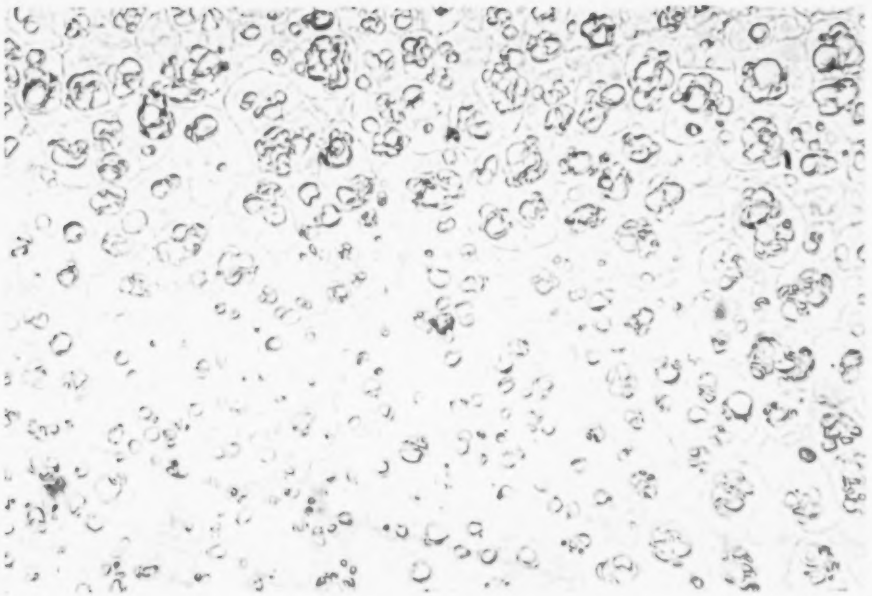
W



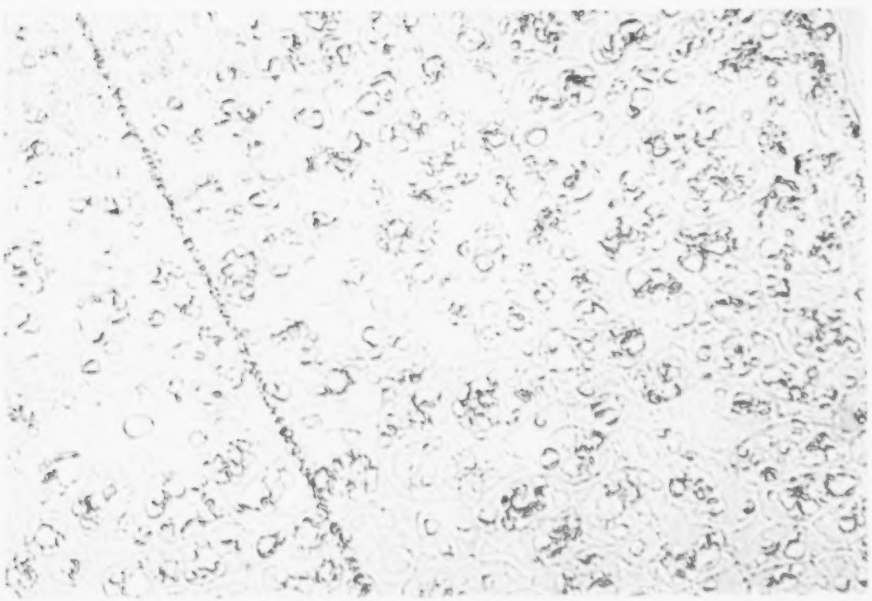
T



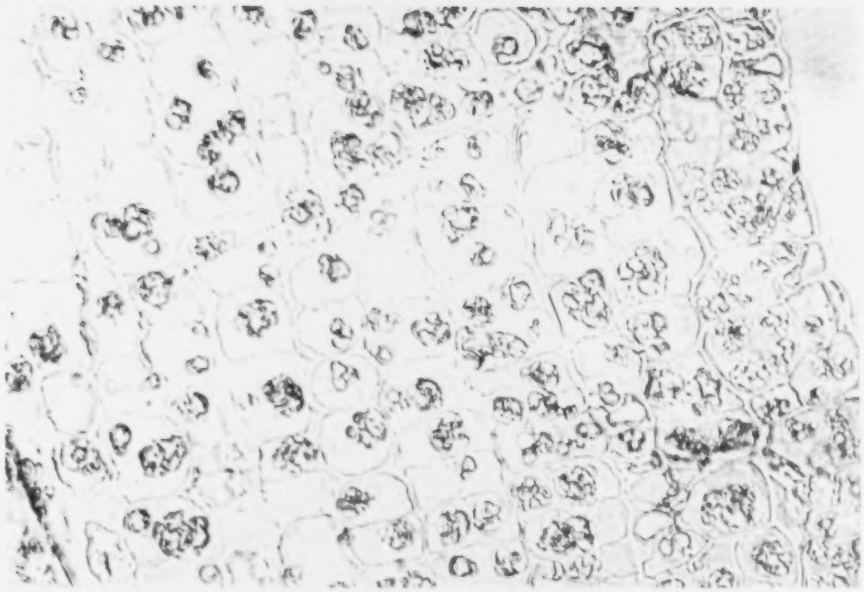
CLO



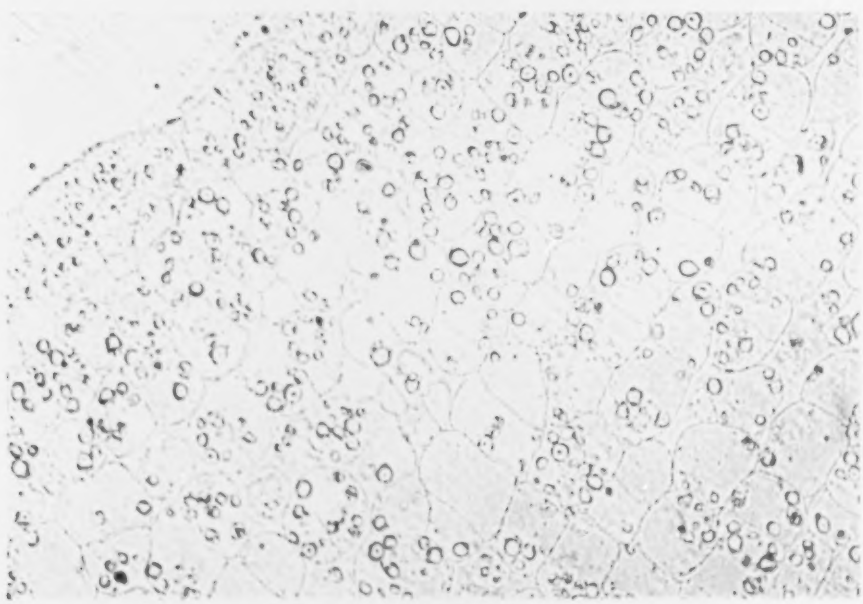
CL7



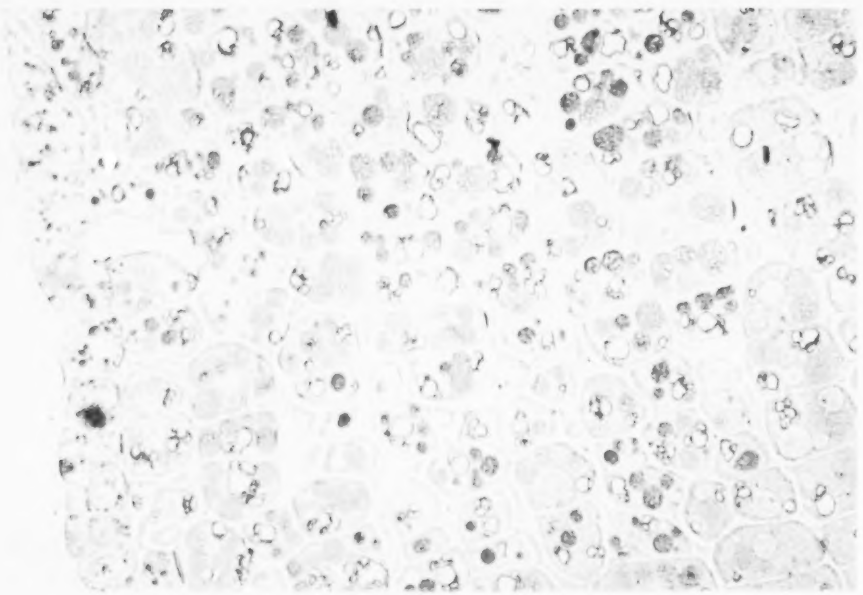
CD0



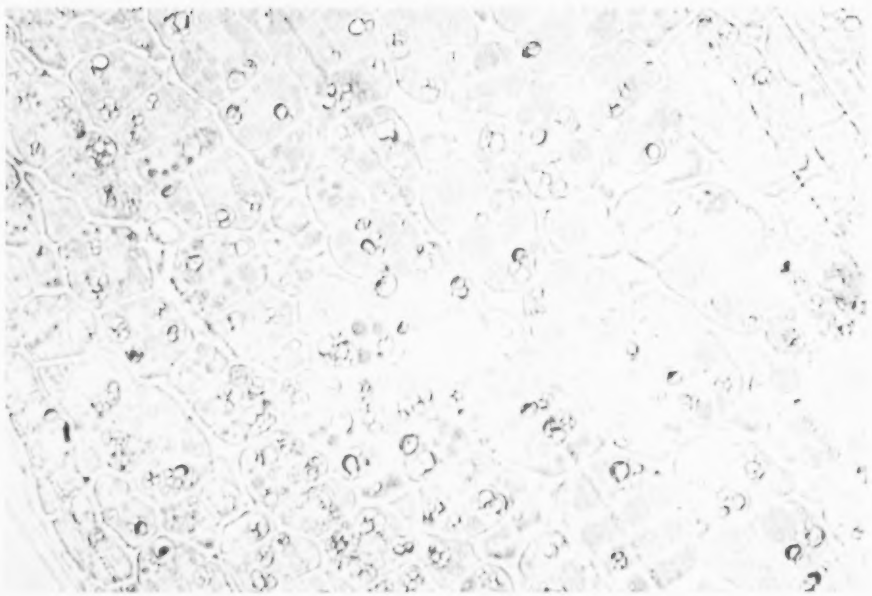
CD7



L+7



DD+7



D+7

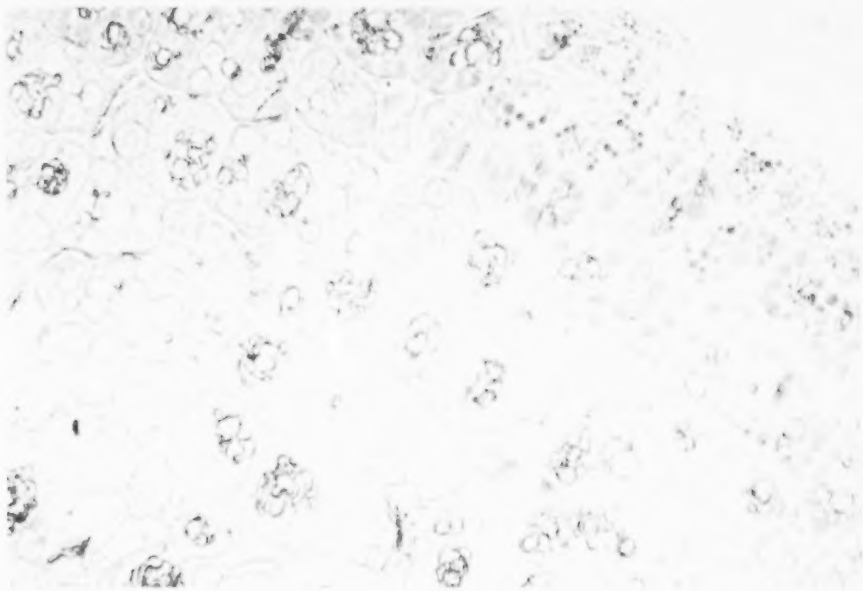
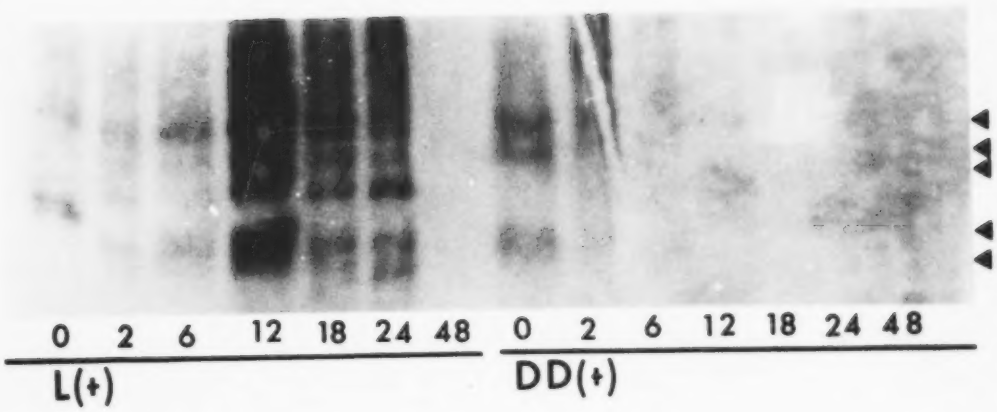


Figure 6-9. Cruciferin protein turnover rate. Cruciferin was purified from pulse-chase labelled MDEs over the course of 48 hours after the chase was initiated to determine the stability of cruciferin subunits in the L(+) and DD(+) culture conditions. 300 000 DPMs were loaded on a gel filtration column and fractions having the same elution as cruciferin were collected and separated on 15% SDS-PAGE according to the methods outlined in materials and methods. The arrows denote the α- and B-subunits of cruciferin.


0 2 6 12 18 24 48 0 2 6 12 18 24 48
L(+)
DD(+)

4.0. Discussion.

During zygotic embryogenesis the accumulation of storage proteins occurs during the cell expansion phase (Crouch and Sussex, 1981; Kermode, 1990; West and Harada, 1993) as embryos go from the torpedo to cotyledonary stage of embryogenesis. Cruciferin and napin begin to accumulate at 23 to 25 DPA (Crouch and Sussex, 1981; DeLisle and Crouch, 1989), with the detection of napin preceding cruciferin by a few days (Murphy *et al.*, 1989; Hoglund *et al.*, 1992). Both proteins undergo a rapid accumulation until 35-42 DPA, then accumulate slowly until seed maturation (Murphy *et al.*, 1989).

Similarly, in the present study, MDEs accumulated cruciferin in the torpedo to cotyledonary stage in the DD(+) and D(+) MDEs and cruciferin and napin in the L(+) MDES. Unlike the zygotic system, the accumulation of cruciferin was observed to precede napin in the L(+) condition. This may reflect differences in the response of the storage protein genes to ABA and/or osmoticum.

Although napin and cruciferin transcripts have been detected in MDEs that have been treated with ABA, little or no storage protein accumulated (Wilen, 1992). Results from the present study also showed little accumulation of the storage proteins when MDEs were cultured in the dark. However, both napin and cruciferin accumulated in MDEs cultured in the light. A short exposure to light as occurred in D(+) MDEs, did not lead to increases in storage protein. Exposure to light during the entire study period (L(+)) led to storage protein levels similar to that in mature dry seed. Therefore, light (80-100 $uEm^{-2}s^{-1}$ from fluorescent and incandescent lights) is required during cruciferin and napin expression for accumulation of the proteins.

In zygotic embryos, the transcripts for both cruciferin and napin are both spatially and temporally regulated (DeLisle and Crouch, 1989; Blundy *et al.*, 1991). Cruciferin transcripts begin to accumulate at 23 DPA, reach maximum levels at 38 DPA, and finally decrease to undetectable

levels in the mature seed (Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989). Napin transcripts are detected at 18 DPA, accumulate to high levels by 27 DPA, and decrease to low levels in the mature, dry seed (Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989; Blundy *et al.*, 1991). Temporal differences in cruciferin and napin expression in MDEs have not been reported previously. In general, the timing of expression was similar in the present study to that observed in zygotic embryos, with cruciferin expression either preceded by or concurrent with napin expression. Further to this, expression was transient. Wilen (1992) was able to show upregulation of transcription by addition of ABA to MDEs cultured in the dark. We, on the other hand, followed steady state transcription from day 0, when the embryos were at the early torpedo stage, then induced transcription transiently over the next 7 days. This was simply a response to culturing in the light.

Osmoticum alone has also been shown to result in increases in napin and cruciferin transcripts (Wilen, 1992). In the present study, both cruciferin and napin transcripts were found to increase in response to osmoticum alone (DD(-), L(-), and D(-) in (Fig. 6-7). According to Wilen (1992) the increases of storage protein transcripts in response to osmotica may be mediated by ABA. Indeed endogenous levels of ABA have been shown to rise when the osmotic potential is increased (Wilen *et al.*, 1990).

There is evidence to suggest that storage protein gene expression is also controlled post-transcriptionally. According to Wilen (1992), neither the application of ABA or treatment with osmoticum led to accumulation of mature cruciferin or napin, despite high levels of transcription. Results from the present study also show low levels of storage proteins despite high levels of transcripts for the proteins in MDEs cultured in the dark.

Unlike previous studies, cruciferin and napin were found to accumulate to levels similar to that in mature seed. This only occurred

in the L(+) embryos. This suggests that in order for post transcriptional and translational events to occur light, ABA, and osmotica are necessary. Since neither storage protein has been shown to accumulate to high levels in MDEs cultured in the presence of ABA and\or osmoticum (Wilen, 1992), it suggests that light may be playing an important role directly or indirectly. These results are supported by the finding of reduced cruciferin protein turnover when cultured in the presence of light (Fig. 6-9).

The failure of storage proteins to accumulate in MDEs could be explained by the lack of protein bodies to house the proteins (Rahman *et al.*, 1992). ABA has been shown cause slow development of a small number of protein bodies protein bodies (Rahman *et al.*, 1992). In the present study, protein bodies developed concurrently with the accumulation of storage proteins. As this was most apparent in MDEs cultured in the light, this suggests that light is required for correct processing and deposition (Fig. 6-8 D).

In the absence of light the storage proteins may be rapidly turned over because of improper processing or exposure to proteases. This was examined in the present study by measuring cruciferin turnover. The results clearly show rapid turnover of cruciferin in the dark, thereby confirming post-transcriptional control of storage protein accumulation. In the L(+) embryos labelled cruciferin increased to maximal levels at 12 hours before declining. Since native cruciferin was purified this suggests that correct processing and deposition of cruciferin occurs in the light from precursor peptides. In the dark the precursor peptides may not be properly processed or deposited and therefore only a small percentage is deposited, resulting in lower levels of cruciferin observed in the DD(+) turnover study. Since *B. napus* is chloryphourous during zygotic embryogenesis, until moisture the content decreases, these effects may be related to photosynthetis. MDEs cultured in the light also green in reponse to the light treatment. Photosynthesis may aid the MDEs

by simply providing more energy, and therefore reduce the energy requirements on other reserves for the accumulation and deposition of cruciferin. However, photosynthesis is probably not directly involved because the D+ embryos are pretreated in the light, undergo greening, but did not accumulate high levels of cruciferin or napin. Therefore, although they should contain the same photosynthetic components as the L+ embryos it did not enhance storage protein accumulation. There is a non-green mutant developed by Dr. Stringham (personal communication) that still accumulates oil and protein, so the effects of photosynthesis are unknown. Furthermore, the present study did not uncouple photosynthetic effects from the observed accumulation of cruciferin and napin in L(+) MDEs. This is needed to be performed in the future to determine whether the effects of light act directly on the accumulation of cruciferin in MDEs or indirectly through photosynthesis or another light regulated product.

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Chapter 7. Overview and Future Directions.

7.1. Introduction.

In northern latitudes the length of the growing season is short. This means there is little time for full sporophytic development, and therefore, plants are often exposed to suboptimal conditions during early and late developmental stages. Species susceptible to low temperature injury during germination display reduced emergence and eventual reductions in crop yield (Pollock and Toole, 1966; Phillips and Youngman, 1971; Hobbs and Obendorf, 1972; Simon, 1979; Stewart *et al.*, 1990). One area for potential improvement lies in understanding the mechanisms involved in seedling emergence during exposure to low temperature. Only then can new avenues be explored in an attempt to improve low temperature emergence, and thereby increase the geographical distribution and growing season.

7.2. Normal emergence.

Emergence has been traditionally, but incorrectly, used to define germination (Bewley and Black, 1994). Emergence is comprised of two developmental processes; germination and early seedling growth. Under the proper conditions successful germination establishes a vigorously growing seedling from a quiescent mature seed. Germination in the strictest sense occurs after hydration of the seed and ends with the developmental processes leading to the elongation of the embryonic axis, usually the radicle (Bewley and Black, 1994). Subsequent growth relies upon the mobilization of the stored reserves to fuel cellular division followed by expansion basipetally in localized regions of the meristems (Finkelstein and Crouch, 1984). In canola, seedling growth is epigeal, therefore extension of the hypocotyl, via cell division and enlargement, forces the cotyledons through the growth matrix (soil) into the air (Esau, 1977). Thereafter, the cotyledons are converted to photosynthesizing leaf-like structures, and support the growth of the plumule (the growing meristem of the shoot) in the presence of light. Further growth of the epicotyl

results in the formation of true-leaf structures and establishes growth of the plant. The cotyledons at this time may remain photosynthetically active or senesce. Events resulting in germination are independent from events associated with early seedling growth. Therefore, successful emergence will only occur if both developmental processes occur in a timely manner, resulting in the establishment of a young seedling. The present study revealed that at low temperature the onset of germination is delayed, and may not occur at lower temperatures. Furthermore, as a consequence seedling growth is also delayed. Further reductions in seedling growth occurred at low temperature and is associated with poor storage reserve mobilization. Emergence could only be imporved if first germination was enhanced at low temperature and the subsequent mobilization of stored reserves and their subsequent utilization was optimized.

7.3. Germination.

Successful germination is species specific, and is dependent upon a number of factors. For instance some seeds require specific environmental or chemical cues to occur prior to germination (Bewley and Black, 1994). Such seeds are referred to as dormant. In these cases dormancy can only be broken when the internal (the state of the seed itself) and external (environmental factors) controls are overcome (Bewley and Black, 1994). In other species, the seed will germinate under appropriate environmental conditions. These seeds are quiescent or non-dormant. Canola is an example of such a plant, as dormancy has been bred out to acquire a more uniform germination (Adler et al., 1993 and references therein).

With respect to low temperature germination some species exhibit chilling resistance where others are chilling sensitive (Hallgren and Oquist, 1990; Bedi and Basra, 1993). Susceptible plants are generaly tropical and subtropical in origin, and include maize (Cal and Obendorf, 1972; Cohn *et al.*, 1979), cotton (Christiansen, 1969), lima bean (Pollock,

1969), field bean (Pollock and Manalo, 1970), rice, cucumber, tobacco (Simon *et al.*, 1976; Herner, 1986; Markowski, 1989 a, b), and soybean (Obendorf and Hobbs, 1970; Hobbs and Obendorf, 1972). Resistant crops include wheat, barley, oats (Bordsworth and Bewley, 1981), *S. alba* (Simon *et al.*, 1976), and *B. napus* (Acharya *et al.*, 1983; Kondra *et al.*, 1983; King *et al.*, 1986; Barber *et al.*, 1991; Wilson *et al.*, 1992; Mills, 1993).

The ability of seeds to germinate at low temperatures is dependent upon a number of factors. These include the species and/or cultivar, the temperature, the duration of the chilling exposure, and phase of germination when the chilling exposure occurs (Bedi and Basra, 1993). Susceptible versus resistant low temperature germination in species appears to differ only in the time required to attain 50% germination (Simon *et al.*, 1976) as demonstrated by Arrhenius plots (Johnson and Thornley, 1985). Species such as *Sinapis alba* (Simon *et al.*, 1976), will germinate at temperatures below 15°C but germination steadily decreases as the temperature is lowered. The lowest threshold for germination is around 2 or 3°C (Bedi and Basra, 1993).

In *B. napus* germination occurs between the temperatures of 2 to 25°C (Canola Growers Manual, 1991). *B. napus* and *B. campestris* cultivars display differences in germination at low temperature (Acharya *et al.*, 1983; Kondra *et al.*, 1983; King *et al.*, 1986; Barber *et al.*, 1991; Wilson *et al.*, 1992; Mills, 1993). The present study reveals that differences also exist within a cultivar during low temperature germination.

Imbibition during low temperature germination may result in chilling injury to the seed (Pollock and Toole, 1966; Pollock, 1969; Bramlage *et al.*, 1979; Bradbeer, 1988; Woodstock, 1988). Damaged seed coats (Tully *et al*, 1981; Taylor and Dickson, 1987) or removal of the seed coat (Ashworth and Obendorf, 1980) can amplify or result in chilling injury during imbibition. The present study employed seed of high quality with little or no damage to the seed coat, thereby minimizing this factor.

Low temperature leads to an increase in the lag phase of water

uptake (Simon *et al.*, 1976; Vertucci and Leopold, 1983; Vertucci, 1989), reduced radicle growth (Christiansen, 1963, 1967; Cal and Obendorf, 1972; Cohn *et al.*, 1979), and lower respiration rates (Woodstock and Pollock, 1965; Simon *et al.*, 1976; Leopold and Musgrave, 1979; Vertucci, 1989). The abnormalities in root growth occur because of the loss of apical control or abortion of the root tip (Bedi and Basra, 1993). Reductions in respiration rates have been correlated with abnormal mitochondrial (Ilker *et al.*, 1979; Chabot and Leopold, 1985; Hodson *et al.*, 1987) and chloroplastic (Krol and Huner, 1984; Krol *et al.*, 1987) development at low temperature. In the present study, germination at 10 and 6°C resulted in an increased lag during phase II of germination, reduced radicle growth and lower respiration rates in comparison to seeds imbibed at 22°C. Therefore, some of the manifestations displayed by susceptible species also occur in canola (a resistant species), but not to the same extent. This suggests that resistant species possess mechanisms to overcome the constraints imposed by exposure to low temperature during germination and early seedling growth.

Imbibition at low temperature results in leakage of cellular components (Pollock and Toole, 1966; Bramalge *et al.*, 1978; Leopold and Musgrave, 1979; Leopold, 1980; Schmidt and Tracy, 1989), and the damage and leakiness displayed by susceptible species is believed to result from the physical stress caused during rapid water uptake (Spaeth, 1989). In the present study the uptake of water was slightly delayed at low temperature, but closely followed the pattern of imbibition displayed by seeds at 22°C. Therefore damage during imbibition is not believed to have played a major role in the delay or reduction observed in germination at low temperature.

7.4. Early seedling growth.

Failure to germinate and initiate subsequent seedling growth at low temperature may result because the cellular membrane integrity is

disrupted and unable to reassemble (Lyons, 1973; Mayer and Marbach, 1981; Raison and Wright, 1983; Bewley and Black, 1994). Another theory involves protein denaturation as the cause of chilling injury in seeds (Simon *et al.*, 1976; Simon, 1979). Chilling temperatures have an effect on cytochrome c oxidase (Maeshima *et al.*, 1980), phosphoenolpyruvate carboxylase (Graham *et al.*, 1979), pyruvate orthophosphate dikinase (Shirakashi *et al.*, 1978), and tubulins from microtubules (Sakiyama and Shibaoka, 1990). The improper function of some of these enzymes could account for the reductions in respiration and the energy required to sustain growth at low temperature.

The amount of cellular leakage was not assessed in the present study, however the developmental imapirments observed in storage protein and oleosin mobilization may suggest that there is some denaturation of the proteins required to carry out these processes and/or a high turnover rate in these proteins.

7.5. Low temperature emergence in *B. napus*.

Despite the fact that emergence is comprised of both germination and early seedling growth, there has been little or no work on these components individually or in concert. Thus, the main objective of this study was to dissect these processes and then recapitulate the events involved in reduced emergence at low temperature in *B. napus*, as follows. Low temperature leads to a temporal delay in germination at 10°C, and both a temporal delay and reduced germination at 6°C. By standardizing thermal time to assess germination on the basis of equivalent heat units, we were able to demonstrate that the delay was a thermal effect. The delay was caused by a prolonged phase II of germination. Reduced germination at 6°C appeared to be caused by a failure of the seed to enter into phase III of germination. ABA was implicated as a causative agent in the delay and reduction in germination. This was supported by results from endogenous ABA analyses and studies in which seed was germinated in ABA or fluridone.

Seedling growth was also affected by low temperature. At 10°C, seedling growth was delayed, but this was attributable solely to the delay imparted by low temperature during germination. At 6°C, seedling growth was compromised by exposure to low temperature. This appeared to be a function of a loss of coordination between the mobilization of storage reserves and the *de novo* synthesis of proteins required for seedling growth and development.

More detailed analyses of the developmental switches from embryogenesis to germination to post-germinative growth indicated again, that the delays at 10°C were imposed by thermal constraints and the reduction in seedling growth at 6°C resulted from both thermal constraints and developmental impairment. At 10°C, post transcriptional modifications were noted, with either high rates of ICL turnover or low rates of translation. The effects at 6°C included poor coordination of lipid mobilization, probably arising from low rates of ICL transcription.

Studies of genotypic and environmental effects suggest that both factors play a role in determination of the potential for low temperature germination and subsequent seedling growth. Previous studies have failed to identify a heritable component. Superior low temperature emergence was not related to seed size, but may be correlated with storage protein content. This prompted further study of storage proteins and their transcripts. As we wished to alter light reaching the embryo, MDEs were utilized. From this portion of the study, we were able to show that light is required for proper coordination of processing and deposition of storage proteins. In the absence of light, transcript levels were high, but little protein accumulated, because of high turnover rates.

Poor seedling emergence at low temperature in *B. napus*, therefore, is a function of temporal delays, caused by thermal constraints and both decreased germination and reduced seedling growth, resulting from poor coordination of developmental processes. These effects can be modified by genotype or by the environment under which the seed matures. As such,

there is scope for numerous approaches to improving low temperature emergence in canola.

7.6. Future directions.

The purpose of this study was to gain insight into some of the underlying biochemical and molecular events occurring during low temperature germination and early seedling growth in *B. napus* cv. Westar. During the course of the current study new avenues of research have resulted. Further research into the endogenous levels of hormones, their functions, and interactions during germination and early seedling growth need to be explored. Then alterations to endogenous levels via the application of hormone synthesis inhibitors or mutant seeds over- or under-expressing vital hormones could be pursued.

As the deposition of storage reserves and/or environmental conditions during embryo maturation may confer superior germination during low temperature, more experimentation is required to study how these factors are related. The development of culture conditions in an artificial embryo system able to accumulate storage proteins may aid in this respect. This system will also enable researchers to uncouple maternal effects delivered through the seed coat or vascular connection during embryogenesis and embryo effects on storage protein deposition. Prior to this however, basic research is required to compare the MDEs to their zygotic counterparts to ensure that the results displayed *in vitro* mimic those exhibited *in vivo*.

Regardless of the direction taken in the attempt to improve low temperature germination in *B. napus*, there is a necessity for basic research on the underlying biochemical and molecular processes. It is obvious that a better understanding of the complex interactions and co-ordination of the developmental processes during germination and early seedling growth is needed. This in turn may enable researchers to discover the rate limiting steps in rapid and synchronous low temperature

emergence. Unfortunately, any means attempted to improve emergence at low temperature may be met with resistance. Nature has been working on this problem, and evolution has selected for the best possible adaptations to the environment. Breeders have accelerated evolution for the benefit of man, but plants' inherent ability to adapt to an ever changing environment may only frustrate mans' desire for change.

7.1. References.

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Appendix.

Polyclonal antibodies directed against cruciferin were employed to follow the degradation of the storage protein over the course of imbibition (Fig. A-1). At the optimal temperature the cruciferin subunits decreased rapidly and were no longer detected by day 6. In addition, cruciferin was not detected in the negative control (lane 1e). At 10°C the levels of cruciferin steadily decreased from 4 to 10 DAI. The 6°C sample displayed only a slight decrease in cruciferin over the same time frame.

By following the breakdown of oleosin (oil body protein) we can indirectly follow the mobilization of lipids. A monoclonal antibody directed against a 20 kD oleosin was observed to decrease readily in the 22°C sample being no longer detectable 10 DAI (Fig. A-2). At 10 and 6°C the oleosin decreased only slightly by 10 DAI. Note, also the absence of oleosin in the negative control, lane 1e.

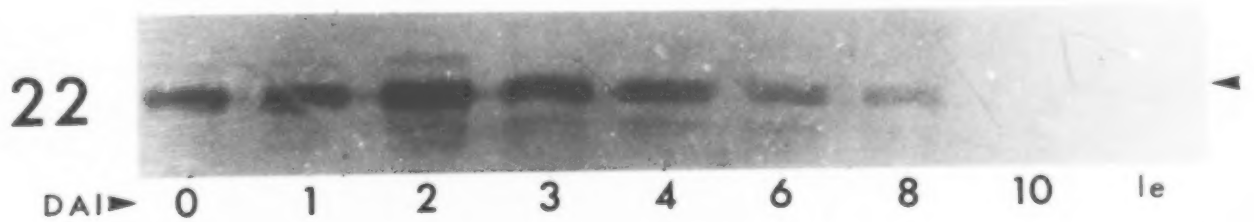
Figure A-1. Western blot analysis of cruciferin from total protein (10 µg/lane) isolated days after imbibition (DAI) during germination and early seedling growth under the optimal and suboptimal temperature conditions in *Brassica napus* cv. Westar. The temperatures tested were 22, 10, and 6°C, with (◂) denoting the 31 and 29 kD α-polypeptides.

22
DAI
0
1
2
3
4
6
8
10
le

10
DAI
1
2
4
6
8
10

6
DAI
1
2
4
6
8
10

Figure A-2. Western blot analysis of oleosin from total protein (1 µg/lane) isolated days after imbibition (DAI), during germination and early seedling growth, under the optimal and suboptimal temperature conditions in *Brassica napus* cv. Westar. The temperatures tested were 22, 10, and 6°C, with (◂) denoting the 20 kD polypeptide.


22
DAI
0
1
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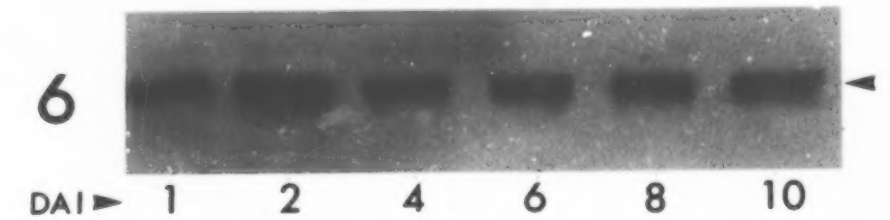
6
DAI
1
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THE END...?